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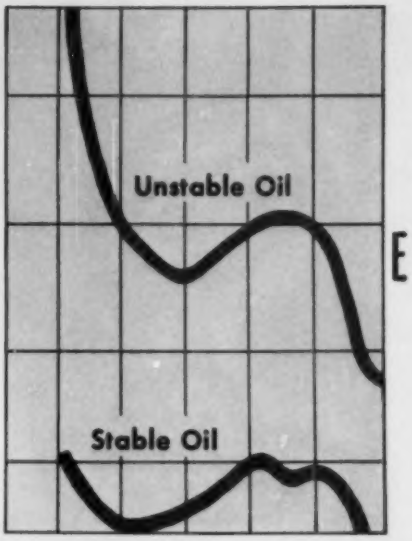

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Some Aspects of the Central Nervous System Activity of Urazole Derivatives II*

By C. L. MITCHELL†, H. H. KEASLING, and C. W. HIRSCHLER

The neurotoxicity, antielectroshock, anti-Metrazol, and antistrychnine activities of another series of urazole compounds have been determined in mice. Depending upon the nature of the substituted nitrogen groups, the members of this series, as with the previous compounds, were found to possess anti-electroshock, anti-Metrazol, antistrychnine, convulsant, and depressant type actions. In general, the urazole compounds are most effective against maximal Metrazol seizures.

THE SIMILARITY of the urazole and hydantoin nucleus prompted an investigation of the C. N. S. activity of a series of substituted urazoles. One group of these derivatives has been reported previously (1). The compounds were relatively short acting and nontoxic. In general, they were found to be most effective against maximal Metrazol seizures. Variations in the substituted nitrogen groups yielded compounds possessing anticonvulsant, convulsant, and depressant type actions.

This report concerns the C. N. S. activity of another series of urazole derivatives and some observations concerning the structure-activity relationship of the C. N. S. actions of the entire series.

EXPERIMENTAL

Adult male albino mice obtained from the Arthur Sutter Farms (Rockland strain), weighing 20 to 25 Gm., were maintained on Purina laboratory chow and allowed free access to food and water except during the test period. The mice were used for one experiment only. The test procedures employed were: (*a*) the supramaximal electroshock seizure technique (M. E. S.), of Toman, *et al.* (2), in which alternating current, 50 ma. of 0.2 second duration was delivered through Spiegel corneal electrodes (3) from an electroshock apparatus constructed according to the design of Woodbury and Davenport (4); (*b*) the maximal Metrazol seizure test (M. M. S.), of Goodman, *et al.* (5), in which Metrazol,[1] 38 mg./Kg., was injected intravenously. The end points for both tests were the abolition of the tonic extensor components of the seizure pattern; and (*c*) ability to prevent death from strychnine, in which 2.5 mg./Kg. of strychnine was injected intraperitoneally (6).

All compounds were suspended in 10% acacia for oral administration. The convulsant stimulus was delivered thirty minutes following urazole administration, with the exception of UR Nos. 103 and 104. The convulsant stimulus for the latter two compounds was delivered one hour (UR 103) and one and one-half hours (UR 104) following drug administration. Those compounds exhibiting protection at 1 Gm./Kg. from electroshock and/or Metrazol were then administered in dosages of 300, 100, and 30 mg./Kg. until less than 50% of the animals were protected.

Acute neurotoxicity (N. T.) was determined by the method of Swinyard, *et al.* (7). The following end points were used: (*a*) positional sense test, (*b*) righting test, (*c*) gait and stance test, (*d*) muscle tone test, and (*e*) equilibrium test. Neurotoxicity was recorded when an alteration in response to any one of the above procedures was observed. The mice were examined at fifteen-minute intervals for at least one hour, and thereafter until a decrease in the number of mice exhibiting neurological symptoms was seen.

The 50% neurotoxic dose (TD_{50}) and the 50% effective dose (ED_{50}) for UR 110 and UR 104 were determined at thirty minutes and one and one-half

* Received June 27, 1959, from the College of Medicine, State University of Iowa, Iowa City.

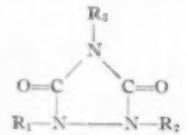
† Smith, Kline and French Fellow in Pharmacology.

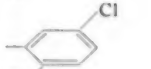
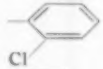
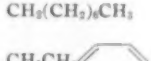
The urazole compounds were kindly supplied by and the work was supported in part by the Knoll Pharmaceutical Co., Orange, N. J., and in part by U. S. P. H. S. Grant B-1079.

The material contained in this report is to be submitted to the Graduate College of the State University of Iowa in partial fulfillment of the requirements for the degree of Ph.D.

[1] The Metrazol was kindly supplied through the courtesy of Dr. R. O. Hauck, Knoll Pharmaceutical Co.

TABLE I.—STRUCTURES OF URAZOLE DERIVATIVES



UR No.	R_1	R_2	R_3
100	H		CH_2CH_3
109	H		$CH_2CH_2CH_3$
111	H		
105	H		
54	H		H
80	CH_3		H
81	CH_3		H
90	CH_3		H
91	CH_3		H
89	CH_3		H
110	CH_2CH_3	CH_2CH_3	H
59	CH_2CH_3		CH_3
58	CH_2CH_3		CH_2CH_3
60	CH_2CH_3		CH_3
69	$HC(CH_2CH_3)_2$		H
63			H
94	$CH_2(CH_2)_4CH_3$		H
92			CH_2OH
85			H
88			H
66			H
70			H
72			H
71	$CH_2(CH_2)_3CH_3$		H
67	$HC(CH_2CH_2CH_3)_2$		H
65			H
101	$CH_2(CH_2)_6CH_3$		H
73	CH_3CH_2 		H
83			H

61	$CH_3CH_2CH_3$		CH_3CH_3	102	$CH_2(CH_2)_7CH_3$		H
62	$CH_3CH_2CH_3$		CH_2CH_2OH	107	$CH_2CH_2CH_2$		H
93	$HC(CH_3)_2$		CH_2OH	108	$CHCH_3$ CH_3		H
57	$CH_2CH{=}CH_2$		H	78	$CH_2(CH_2)_6CH_3$		H
64	$CH_2CH_2CH_2CH_3$		H	103	$CH_2(CH_2)_9CH_3$		H
56	$CHCH_2CH_3$ CH_3		H	104	$CH_2(CH_2)_{10}CH_3$		H
79	$CH_2CH{=}CHCH_3$ CH_3		H	77	$HC(\text{})_2$		H
75	$CH_3C{=}CH_2$ $CHCH_2CH_2CH_3$ CH_3		H				
90			H				

hours, respectively. Similar tests for trimethadione,[2] phenobarbital, and diphenylhydantoin[3] were determined at one and one-half hours, two hours, and three hours, respectively. These time intervals represent the approximate time of peak effectiveness for each drug, as determined in our laboratories utilizing the M. E. S. technique. The time of peak effect for the standard anticonvulsants is in agreement with that reported by Swinyard, *et al.* (7), and Chen, *et al.* (8).

The ED_{50} in the Metrazol seizure threshold test (Met.) of Swinyard (7), was also determined for the above compounds. In this test, Metrazol, 85 mg./Kg. was injected subcutaneously; the end point was the abolition of clonic spasms. In the Met. test, the Metrazol was injected subcutaneously ten minutes prior to the time of peak effect and the mice were observed for a period of thirty minutes.

The computations for fitting the probit-log dose regression line, the determination of the TD_{50}, ED_{50}, protective index (P.I.), and the 95% confidence interval were done according to the methods described by Finney (9). In determining the ED_{50} at least three dose levels, yielding between 10 and 90% effect were used. At least 10 mice were utilized in determining each point.

The time of peak anticonvulsant effect and duration of action of one compound (UR 104) was determined by the M. M. S. test. A dose of 100 mg./Kg. was administered to five groups of 10 animals. The respective groups of mice were then injected with Metrazol at thirty, sixty, ninety, one hundred and twenty, and one hundred and eighty minutes.

The sleeping time produced by two compounds (UR 75 and 96) was compared with hexobarbital. Sleeping time was recorded as the time interval in which the righting reflex of the mice was absent. The effect of five compounds (UR 73, 83, 107, 108, and 109) upon the sleeping time of hexobarbital was also determined. The convulsant compounds were administered orally, two minutes prior to the i. p. injection of hexobarbital. The 95% confidence intervals were calculated according to the method described by Snedecor (10).

RESULTS

The structures of the urazole derivatives studied are detailed in Table I. UR Nos. 109, 73, 83, 107, and 108, which were convulsants, produced death in 50% or more of the mice at 1 Gm./Kg. Less than 50% of the mice were killed at 1 Gm./Kg. with the following compounds: UR Nos. 62, 93, 94, 111, 59, 58, and 88. The remaining compounds were relatively nontoxic at 1 Gm./Kg. (i. e., no mice were killed). The predominant neurotoxic effect was ataxia.

The anticonvulsant activities are detailed in Table II. Data for phenobarbital, diphenylhydantoin, and trimethadione have been reported previously (1) and are in general agreement with those of Swinyard, *et al.* (7), Goodman, *et al.* (5), and Chen, *et al.* (8). As with the previously studied materials (1), the present urazole compounds possess relatively good anti-M. M. S. activity, and they are

[2] The trimethadione was kindly supplied through the courtesy of Dr. R. K. Richards, Abbott Laboratories.
[3] The diphenylhydantoin was kindly supplied through the courtesy of Dr. Graham Chen, Parke, Davis and Co.

ineffective in the M. E. S. test in non-neurotoxic doses.

Neurotoxicity tests indicated that all active members of this series were rapidly absorbed from the intestinal tract and with the exception of UR Nos. 103, 104, and 110, had a relatively short duration of action. All of the urazole derivatives reported previously (1) appeared to have a short duration of action. Time duration studies on UR 104, 100 mg./Kg. (Fig. 1), indicate that this compound has a peak effect ninety minutes after oral administration. Some anticonvulsant activity is still present three hours after administration. A similar study of UR 110 revealed a peak effect at thirty minutes.

The TD_{50} and the ED_{50}'s in the M. M. S., Met., and M. E. S. tests for two of the more active members of the series (UR 104 and UR 110) were determined. A comparison of these data with those of trimethadione, phenobarbital, and diphenylhydantoin are presented in Table III. From the data presented, it would appear that UR 104 and UR 110 are relatively specific antagonists of Metrazol.

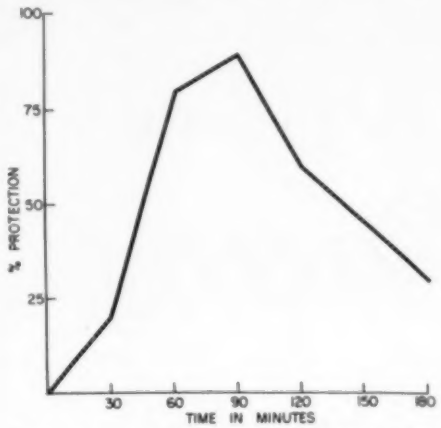


Fig. 1.—Duration of action of UR 104, 100 mg./Kg.

TABLE II.—ANTICONVULSANT ACTIVITY OF URAZOLES[a,b,c]

UR No.	Test	1,000	300	100	30	UR No.	1,000	300	100	30
111	NT[d]	15/20	..	..	..	..	20/20	20/20	5/20	0/10
	MES[e]	0/10	..	..	..	96	10/10	10/10	0/10	..
	MMS[f]	0/10	..	..	..	..	10/10	9/9	6/10	2/9
80	NT	20/20	20/20	0/10	..	..	20/20	5/20	0/10	..
	MES	10/10	1/10	..	..	92	7/9	0/9	..	..
	MMS	8/8	10/10	2/8	..	..	9/9	8/9	0/10	..
81	NT	16/16	9/20	..	..	..	0/20	..	..	..
	MES	4/8	1/10	..	..	85	0/8	..	..	..
	MMS	8/8	3/9	..	..	..	8/8	5/10	1/10	..
90	NT	20/20	10/10	0/10	..	..	20/20	11/20	0/10	..
	MES	3/9	..	..	..	88	5/8	2/10	..	..
	MMS	9/9	7/7	4/10	..	..	8/8	7/9	6/10	2/10
91	NT	20/20	0/10	0/10	..	..	12/20	4/10	2/10	..
	MES	8/8	0/10	..	..	66	8/9	0/10	..	..
	MMS	7/7	9/10	1/10	..	..	5/6	5/8	4/10	..
89	NT	0/20	..	..	..	..	0/20	..	..	..
	MES	0/9	..	..	..	70	0/10	..	..	..
	MMS	3/9	..	..	..	..	7/10	0/9	..	..
110	NT	6/10	1/20	0/10	..	..	6/20	..	..	..
	MES	0/10	..	..	..	72	0/10	..	..	..
	MMS	10/10	8/10	6/10	2/10	..	8/9	1/10	..	..
59	NT	20/20	0/20	..	..	..	20/20	7/18	0/10	..
	MES	9/9	0/10	..	..	71	6/10	0/8	..	..
	MMS	8/8	4/9	..	..	..	9/10	4/8	0/10	..
58	NT	20/20	0/10	..	..	..	1/20	0/10	..	..
	MES	1/10	..	..	..	67	3/9	..	..	..
	MMS	5/10	1/10	..	..	..	7/8	3/10	..	..
60	NT	20/20	0/10	..	..	..	8/20	2/10	..	..
	MES	3/10	..	..	..	65	0/10	..	..	..
	MMS	7/7	1/8	..	..	..	9/9	3/8	..	..
61	NT	11/17	0/10	..	..	..	20/20	1/10	0/10	..
	MES	1/10	..	..	..	78	0/10	..	..	..
	MMS	5/7	2/9	..	..	..	9/9	8/9	5/10	1/10
62	NT	20/20	7/20	..	..	..	20/20	0/10	0/10	..
	MES	10/10	4/10	..	..	103	2/10	..	..	..
	MMS	9/9	4/9	..	..	..	10/10	9/10	2/10	..
93	NT	20/20	17/20	0/10	..	..	20/20	10/10	0/10	..
	MES	8/9	3/10	..	..	104	2/10	..	..	..
	MMS	10/10	9/9	4/9	..	..	10/10	10/10	8/10	2/10
75	NT	19/19	10/10	0/10	..					
	MES	8/8	2/10	..	..					
	MMS	8/8	10/10	7/10	1/7					

[a] UR Nos. 100, 105, 54, and 77 inactive at 1 Gm./Kg.
[b] UR Nos. 109, 73, 83, 107, and 108 are convulsant (see text).
[c] UR Nos. 57, 64, 56, 79, 75, 96, 69, 63, 94, 101, and 102 stimulate, then depress at 1 Gm./Kg. (see text).
[d] NT = Neurotoxicity (see text).
[e] MES = Supramaximal electroshock seizure technique (see text).
[f] MMS = Maximal Metrazol seizure test (see text).

TABLE III.—TOXICITY, ANTI-M. M. S. POTENCY, ANTI-MET. POTENCY AND ANTI-M. E. S. POTENCY FOR UR 104, 110, TRIMETHADIONE, PHENOBARBITAL, AND DIPHENYLHYDANTOIN[a]

Drug	Time,[b] Min.	Neurotoxicity, TD_{50}, mg./Kg.	M. M. S. Potency, ED_{50}, mg./Kg.	Met. Potency, ED_{50}, mg./Kg.	M. E. S. Potency, ED_{50}, mg./Kg.
UR 104	90	204 (55–684)	55 (35–75)	88 (46–131)	820 (638–989)
UR 110	30	875 (702–1099)	80 (30–130)	273 (169–422)	>1000
Trimethadione	90	975 (760–1390)	236 (196–281)	421 (275–556)	1082 (923–1262)
Phenobarbital sodium	120	60 (47–77)	9 (6–12)	27 (17–36)	21 (17–25)
Diphenylhydantoin sodium	180	100 (56–184)	11 (9–12)	Ineffective in doses up to 100 mg./Kg.	14 (11–17)

[a] Values in parentheses represent the 95% confidence interval.
[b] Time elapsed after administration of drug before Metrazol was injected.

Compounds No. 73, 83, 107, 108, and 109 produced convulsions at 1 Gm./Kg. With the exception of UR 109, the convulsions were primarily clonic in nature. Tonic flexion was the most pronounced effect produced by UR 109. All of the compounds produced convulsions within ten minutes. UR Nos. 73, 83, 107, and 108, at 1 Gm./Kg. produced death in all of the animals within one hour after administration. At 300 mg./Kg., UR 73 and UR 83 produced periodic mild clonus which lasted for approximately one and one-half hours. Mice receiving these two compounds in a dose of 100 mg./Kg. appeared hyperactive. UR 107, 300 mg./Kg. produced death in three out of ten animals, mild stimulation was noted from 100 mg./Kg. UR 108, 300 mg./Kg. produced death in all animals. At 100 mg./Kg., hyperactivity was noted. UR 109, 1 Gm./Kg., produced death in four out of 10 animals within one and one-half hours after administration. No effect was seen from this compound at 300 mg./Kg. The effect of these compounds on the sleeping time of mice receiving 150 mg./Kg. of hexobarbital administered i. p. is detailed in Table IV. UR 107, 100 mg./Kg., appeared to prolong the sleeping time of mice receiving hexobarbital. The other compounds, in the dose studied, had no apparent effect.

Compounds No. 57, 64, 56, 79, 75, 69, 63, 94, 101, and 102, 1 Gm./Kg., caused initial stimulation which was followed by depression. However, only UR 75 and UR 96 induced a loss of righting reflex at 400 mg./Kg. Table V shows the mean sleeping time of UR 75, and UR 96, 400 mg./Kg., as compared to a similar dose of hexobarbital.

All compounds (except UR 100 and UR 105) were tested at a dose of 1 Gm./Kg. for their ability to protect against death produced by i. p. injection of 2.5 mg./Kg. of strychnine. Only UR Nos. 80, 81, 90, and 89 provided significant protection. The activity of these compounds in comparison with 250 mg./Kg. of Zoxazolamine[c] and 500 mg./Kg. of mephenesin is detailed in Table VI. It would appear that UR 80 and UR 81, 250 mg./Kg., are comparable to Zoxazolamine.

TABLE IV.—EFFECT OF UR 73, UR 83, UR 107, UR 108, AND UR 109 UPON THE MEAN SLEEPING TIME OF HEXOBARBITAL, 150 MG./KG. I. P. IN THE MOUSE

Hexobarbital, 150 mg./Kg. i. p. With:	Mean Sleeping Time, Min.	95% Confidence Interval
Nothing	51	36–65
UR 73 100 mg./Kg. (oral)	63	46–80
UR 83 100 mg./Kg. (oral)	71	54–88
UR 107 100 mg./Kg. (oral)	138	77–200
UR 108 100 mg./Kg. (oral)	83	56–116
UR 109 500 mg./Kg. (oral)	68	55–81

TABLE V.—MEAN SLEEPING TIME OF UR 75, UR 96, AND HEXOBARBITAL, 400 MG./KG., ORALLY

Drug	Mean Sleeping Time, Min.	95% Confidence Interval
UR 75	19	1–36
UR 96	22	3–40
Hexobarbital	41	33–48

TABLE VI.—ABILITY TO PREVENT DEATH FROM I. P INJECTION OF 2.5 MG./KG. OF STRYCHNINE

Drug	Oral Dose	Time,[a] Min.	No. of Animals Surviving/ No. of Animals Tested
UR 80	250 mg./Kg.	30	4/10
UR 81	250 mg./Kg.	30	6/10
UR 90	1 Gm./Kg.	30	3/10
UR 89	1 Gm./Kg.	30	6/10
Zoxazolamine	250 mg./Kg.	60	4/10
Mephenesin	500 mg./Kg.	5	3/10

[a] Time elapsed after administration of drug before strychnine was injected.

DISCUSSION

In our previous report (1), factors validating a discussion of the structure-activity relationship of the urazole derivatives were enumerated and the following observations were made: (*a*) at least one phenyl group on the adjacent nitrogen atom (R_1 or R_2, Table I) is essential for anticonvulsant activity; (*b*) if R_1 or R_2 is phenyl, all other substituents except hydrogen on the adjacent nitrogen can confer anticonvulsant activity; and (*c*) the R_3 substituent must be small or relatively polar for maximal anticonvulsant activity.

The results obtained in the present series indicate that while a phenyl group on one of the adjacent ni-

[c] The Zoxazolamine was kindly supplied through the courtesy of Dr. Charles F. Kade, Jr., McNeil Laboratories, Inc.

trogen atoms is sufficient for anticonvulsant activity, it is not necessary, since UR 110 (1,2-diethyl urazole) was one of the more active compounds in this series. This was the only compound, however, which possessed anticonvulsant activity and did not contain a phenyl group on one of the adjacent nitrogen atoms.

In this series, when R_1 or R_2 is phenyl, most of the groups except hydrogen do confer anticonvulsant activity (compare UR 81, 60, 61, 93, 57, 64, 56, 79, 75, 96, 69, 63, 94, 92, 66, 72, 71, 67, 65, 101, 102, 78, 103, and 104, with UR 100, 109, 111, and 105). However five compounds (UR 73, 83, 107, 108, and 77) did not possess anticonvulsant activity. All of these compounds, except UR 77 (which was inactive at 1 Gm./Kg.) were convulsants.

Although the number of comparisons in this series is limited, the results would seem to substantiate the conclusion that the R_3 substituent must be small or relatively polar for maximal anticonvulsant activity (compare UR Nos. 59 and 58, UR 62 and 61).

UR Nos. 92, 85, 88, 66, 70, and 72 are derivatives of one of the more active anticonvulsant urazole compounds reported previously (UR 20), 1-phenyl-2-cyclohexyl urazole) (1). Compounds No. 92, 85, 70, and 72 are less active than UR 20. UR 88 and UR 66 are approximately equal to UR 20 in anticonvulsant activity. However, UR 88 appears to be more neurotoxic than UR 20.

UR Nos. 80, 81, 90, 91, and 89 are derivatives of the only compound (UR 8, 1 phenyl-2-methyl urazole) reported previously (1) which possessed significant antistrychnine activity. These compounds were the only members of this series which possessed antistrychnine activity. UR 80 and UR 81 are more active than UR 8, while UR 90 and UR 89 are approximately equal in activity to UR 8. UR 91, 1 Gm./Kg., was devoid of antistrychnine activity.

In this series, as in the urazole derivatives reported previously, a variety of actions was noted. The anti-Metrazol activity, however, appears to be one of the more striking properties of the urazoles.

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The Physical Chemical Evidence for Aspirin Anhydride as a Superior Form for the Oral Administration of Aspirin*

By EDWARD R. GARRETT

Physical chemical and kinetic investigations show that aspirin anhydride is a source of aspirin and that it should not produce digestive upset because of salicylic acid production or undissolved aspirin that may adhere to the gastric mucosa and irritate. On oral ingestion, aspirin anhydride should give higher acetylsalicylate blood levels than aspirin alone due to the latter's pH-dependent intestinal absorption. Complete equations are presented for aspirin anhydride's homogeneous and heterogeneous hydrolysis and solubility and the specific equations for physiological conditions have been derived. Procedures have been developed for the assay of aspirin anhydride and all probable derivatives.

THE MECHANISM of the analgetic action of acetylsalicylic acid, aspirin, has been subject to much discussion. The possible side effects are of widespread interest. Without introducing an excessive review or critique of the literature, several rational opinions based on experimental evidence may be stated.

The onset and duration of aspirin concentration in the blood can be correlated with analgetic relief and this action may be expressed mainly by the unhydrolyzed acetylated fraction in the plasma (1, 2). Aspirin can cause gastric irritation, nausea, and even gastrointestinal bleeding in a considerable number of people (3–6). Disturbances from acetylsalicylic acid are reported to be less serious than from salicylic acid (7), and annoyances of the former have been ascribed to the immediate area surrounding particles (4). Here, irritation could be ascribed to the acid function. There is evidence that soluble material is less irritating (6).

It rationally follows that if a form of "aspirin" could produce equivalent aspirin blood levels and less salicylic acid than acetylsalicylic acid, the

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net result could be less gastric irritation without lessened analgetic acitivity. Further, if this "aspirin" were nonacidic and produced higher aspirin blood levels, it might even be found to be superior to acetylsalicylic acid as the optimum form for administration of this drug.

This paper presents and considers the physicochemical and kinetic investigations on aspirin anhydride which permit consideration of it as a superior "aspirin."

EXPERIMENTAL

When the aspirin or salicylic acid impurities of aspirin anhydride (AA) were to be determined quantitatively simultaneously with the rate study for the determination of its heterogeneous hydrolysis, 100 mg. AA were accurately weighed into a 15 × 45-mm. glass vial (Kimble Opticlear) with a press fit polyethylene cap. Four milliliters of 0.1000 *N* HCl were pipetted into the vial. The HCl had been previously maintained at the temperature of the study. The vials were strapped horizontally on a bar suspended vertically into a constant temperature bath. The upper portion of the bar was clamped on the horizontal arm of a Burrell wrist-action shaker; the lower end of the bath was free. The net action of the vials was elliptical and gave excellent agitation of the dispersed AA in the liquid. Quantitative weighing of AA was not necessary when only solubility or heterogeneous rate were to be determined.

The AA was filtered from the solution by a sintered-glass filter (medium).

In run 1 (see Table I and Fig. 1) both filtrate and residue were assayed spectrophotometrically for AA content by the following procedure. Two ml. of filtrate, or 3.5 mg. residue, were made up to 25 ml. with dioxane, i. e., solution *A*. To 10 ml. of solution *A* were added 10 ml. 0.250 *M* HCl and the final volume, *B*, was made up to 25 ml. with dioxane. Also, to 10 ml. of solution *A* were added 10 ml. 0.100 *M* sodium acetate and the final volume, *B'*, was made up to 25 ml. with dioxane. Spectra of both solutions, *B* and *B'*, were run immediately on the Cary spectrophotometer against the appropriate blanks. Typical Cary model 11 curves for aspirin anhydride, salicylic acid, and aspirin are given in Fig. 2.

No significant change was observed in the spectra of aspirin anhydride within ten minutes when in such solutions.

Since the background absorbance of the aqueous dioxane changes with time, especially when in contact with HCl, the spectrophotometric blanks were prepared with the same dioxane at the same time of sample preparation. The dioxane used was distilled on the same day of use and the initial fractions were discarded. The remaining dioxane was purged with nitrogen and not permitted to stay in contact with any aqueous solvent for any great interval.

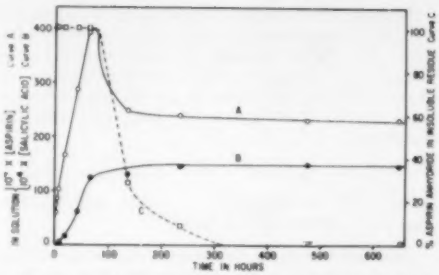


Fig. 1.—Effect of time of heterogeneous hydrolysis on the concentrations of aspirin anhydride and its hydrolytic derivatives. The aspirin anhydride, 100 mg., was equilibrated in 4 ml. 0.1 *N* HCl at 25.8°. Curve A represents the molar concentration of aspirin and curve B that of salicylic acid in solution. Curve C is the % aspirin anhydride in the undissolved portion.

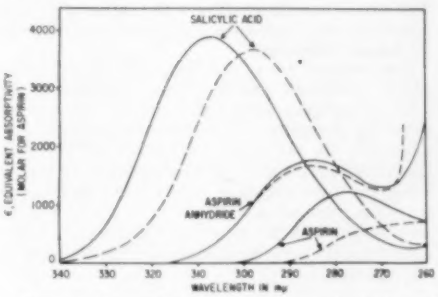


Fig. 2.—Absorptivities of aspirin anhydride and hydrolytic derivatives in 60% (by vol.) dioxane. The solid curves are spectra in 0.1 *N* HCl. The dashed curves are spectra in 0.1*N* sodium acetate.

TABLE I.—ESTIMATION OF SOLUBILITY OF ASPIRIN ANHYDRIDE [AA]$_s$ AT VARIOUS TEMPERATURES IN AQUEOUS SOLUTION (100 MG. IN 4 ML. 0.1000 *N* HCl)

Run	Compd.	°C.	10^4k (sec^{-1}) In 10% Dioxane[a]	10^4k (sec^{-1}) In 0% Dioxane[b]	Slope $10^4d[\text{asp}]+[\text{sal}]/dt$ (M/L./sec.)	Intercept $10^4[\text{asp}]_s$	Intercept $10^4[\text{AA}]_s$
1	1	25.8	8.03	20.0	1.85	67	0.457
2	2	25.7	8.02	20.0	1.83	0	0.463
3	3	30.3	9.65	24.1	2.50	4	0.520
4	2	40.1	16.0	40.0	8.81	0	1.101
5	2	50.4	25.4	63.5	24.9	0	1.961

[a] The variation of k in 0.1000 *N* HCl, 10% dioxane, had been previously determined as a function of temperature (8), where $\log k = 1.960/T + 3.46$ and where $T = 273 + °C$.

[b] The effect of per cent dioxane on k at 25° is linear in the range 0–10% dioxane, i. e., $k = -0.0436$ [per cent dioxane] + 0.3 (8). It was assumed that the effect of decreasing dioxane content on k was the same for all temperatures so that $k^{25.7°}_{0\%\text{ dox.}} / {}^{25.7°}_{10\%\text{ dox.}} \times k^{T°}_{10\%\text{ dox.}} = k^{T°}_{0\%\text{ dox.}}$, where the former quotient is 20/8.

Several three-component assays gave no indication of any significant amount of AA in the filtrate. Thus the aspirin and salicylic acid content of the filtrate was calculated from the absorbances, A, at 307.5 mμ and 277.5 mμ in the HCl-dioxane solutions of the Cary model 11 using the following equation:

$$[\text{aspirin}] = (A_{277.5} - \epsilon_{sal}^{277.5}[\text{sal}])/\epsilon_{asp}^{277.5} \quad \text{(Eq. 1)}$$

where $\epsilon_{sal}^{277.5} = 805$, $\epsilon_{asp}^{277.5} = 1{,}210$, and $[\text{sal}] = A_{307.5}/\epsilon_{sal}^{307.5}$, where $\epsilon_{sal}^{307.5} = 3{,}890$.

The aspirin anhydride content (AA) of the residue was calculated from the absorbances, A, in the HCl-dioxane solutions on the Cary model 14 using the following equation:

$$[\text{AA}] = \frac{\epsilon_{asp}^{270}[A_{297} - \epsilon_{sal}^{292}A_{320}/\epsilon_{sal}^{320}] - \epsilon_{asp}^{292}[A_{276} - \epsilon_{sal}^{276}A_{320}/\epsilon_{sal}^{320}]}{\epsilon_{AA}^{292}\epsilon_{asp}^{270} - \epsilon_{asp}^{292}\epsilon_{AA}^{270}} \quad \text{(Eq. 2)}$$

where $\epsilon_{asp}^{270} = 1{,}200$, $\epsilon_{sal}^{292} = 2{,}700$, $\epsilon_{sal}^{320} = 1{,}898$, $\epsilon_{asp}^{292} = 250$; $\epsilon_{AA}^{292} = 1{,}490$, and $\epsilon_{AA}^{270} = 1{,}540$, where $\epsilon^{\lambda}{}_{AA}$ is the equivalent absorptivity = 0.5 × molar absorptivity for aspirin anhydride.

The composition of the filtrate in all other runs at 25.7, 30.3, 40.1, and 50.4° was determined on the basis of negligible anhydride content and use of the following procedure. Two milliliters of filtrate were diluted to 25, 50, or 100 ml. with 0.1000 N HCl, the amount dependent on the magnitude of the absorbance of the dissolved aspirin and salicylic acid. Readings were taken on the Cary model 11 at 277.5 and 394 mμ and the amounts of aspirin, [asp], and salicylic acid, [sal], were calculated from the following equations:

$$[\text{sal}] = A_{394}/\epsilon_{sal}^{394} \quad \text{(Eq. 3)}$$

where $\epsilon_{sal}^{394} = 3{,}524$, and

$$[\text{asp}] = (A_{277.5} - \epsilon_{sal}^{277.5}[\text{sal}])/\epsilon_{asp}^{277.5} \quad \text{(Eq. 4)}$$

where $\epsilon_{sal}^{277.5} = 1{,}025$ and $\epsilon_{asp}^{277.5} = 1{,}128$.

RESULTS

Since aspirin anhydride in homogeneous aqueous solution undergoes pseudo first-order hydrolysis to aspirin which may subsequently hydrolyze to salicylic acid (8, 9), the hydrolysis of aspirin anhydride in saturated solution should be apparent zero order (10) and

$$d[\text{AA}]/dt = -k[\text{AA}]_s = \text{constant} \quad \text{(Eq. 5)}$$

where $d[AA]/dt$ is in moles of aspirin anhydride hydrolyzed to aspirin per liter per second, k is the pseudo-first order rate constant in homogeneous solution (sec.$^{-1}$) and $[\text{AA}]_s$ is the solubility of anhydride in moles per liter.

Since

$$-d[\text{AA}]/dt = (1/2)d\{[\text{asp}] + [\text{sal}]\}/dt \quad \text{(Eq. 6)}$$

the knowledge of the rate of appearance of aspirin and salicylic acid will permit the evaluation of $k[\text{AA}]_s$ and knowledge of k will permit estimation of the solubility of $[\text{AA}]_s$ in saturated solution. It follows that the plots of [asp] + [sal] *vs.* time at a constant temperature and pH should be linear. Such plots are given in Figs. 3 and 4 for the data.

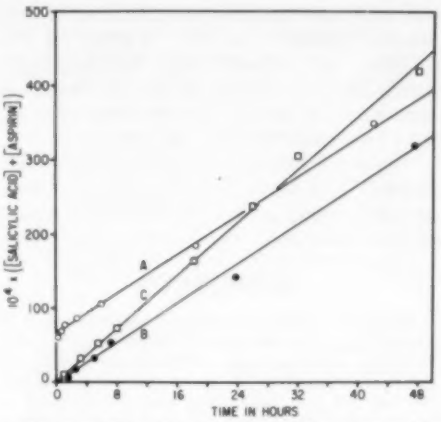


Fig. 3.—Effect of aspirin contaminant of aspirin anhydride on plot of hydrolytic products as a function of time for 100 mg. per 4 ml. 0.1 N HCl. Curve A is for compound 1 at 25.8°; curve B is for compound 2 at 25.7°; and curve C is for compound 3 at 30.3° (see Table I).

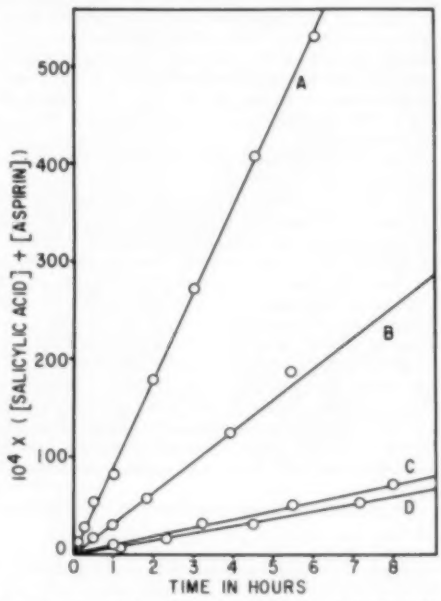


Fig. 4.—Plot of concentrations of hydrolytic products of aspirin anhydride in saturated 0.1 N HCl solution as a function of time. Curve A is at 50.4°; curve B is at 40.1°; curve C is at 30.3°; and curve D is at 25.7°.

The slopes so determined along with the k values from homogeneous hydrolysis permit estimation of the solubility, $[\text{AA}]_s$, as given in Table I.

The intercept provides an independent estimate of the amount of aspirin contaminant. In this regard, this method can serve as an analytical procedure. The compound 1 has 4.8% aspirin; the compound 2 has 0.0% aspirin; and the compound

3 has 0.3% aspirin if the three, six, and eight hours alone are used (see Fig. 3).

The dependence of anhydride solubility on temperature may be expressed by:

$$[AA]_s = P_s e^{-\Delta H_s/RT} \qquad \text{(Eq. 7)}$$

where ΔH_s is the heat of solution in Kcal./mole, R is the gas constant (1.987 calories degree^{-1} mole^{-1}) and T is the absolute temperature. Similarly, the dependence of heterogeneous hydrolysis rate may be expressed by:

$$\begin{aligned} d[AA]/dt &= k[AA]_s = P e^{-\Delta H_T/RT} \\ &= P_a e^{-\Delta H_a/RT}\, P_s e^{-\Delta H_s/RT} \\ &= (P_a P_s) e^{-(\Delta H_a + \Delta H_s)/RT} \end{aligned} \qquad \text{(Eq. 8)}$$

where ΔH_a is the heat of activation for the homogeneous hydrolysis of aspirin anhydride in 0.1000 N HCl.

The logarithmic forms of Eqs. 7 and 8 are plotted in Fig. 5 as based on the data of Table I. The results are tabulated in Table II.

Thus, the heat of solution (ΔH_s) for aspirin anhydride is 11.2 Kcal./mole where the apparent heat of activation (ΔH_T) for the pseudo-zero order heterogeneous hydrolysis is 20.5 Kcal./mole. The heat of activation for the homogeneous hydrolysis of aspirin anhydride is $\Delta H_a = \Delta H_T - \Delta H_s = 9.3$ Kcal./mole.

It has been previously shown in studies on homogeneous hydrolysis that the ΔH_a in 0.1 N HCl, 10% dioxane, was 9.9 Kcal./mole (8). The above calculation is confirmatory.

TABLE II.—DATA ON TEMPERATURE DEPENDENCE OF ASPIRIN ANHYDRIDE SOLUBILITY, $[AA]_s$ IN M/L., AND RATE OF ANHYDRIDE HYDROLYSIS, $-d[AA]/dt$ IN M/L./SEC., IN 0.1 N HCl

Dependent	S[a]	log P	ΔH (Kcal./mole)
$[AA]_s$	2,445	3.845	11.2
$d[AA]/dt$	4,475	7.927	20.5
k	2,030[b]	4.082[b]	9.3[b]

[a] Slope of plots in Fig. 4 for logarithmic form of Eqs. 7 and 8, log (dependent variable) $= -S/T + \log P = -\Delta H/2.303R(1/T) + \log P$.
[b] Calculated from the difference of the above two values.

CALCULATIONS

The equations for the prediction of hydrolysis of saturated solutions of aspirin anhydride under any conditions of pH, acetate buffer concentration, and temperature are given in the pseudo-zero order Eqs. 5 and 6 where

$$k = k_o + k_{OH^-}[OH^-] + k'_{Ac}[C_2H_3O_2^-] \qquad \text{(Eq. 9)}$$

$$k_o = 10^{4.082} 10^{-2,030/T} \qquad \text{Eq. 10)}$$

$$k_{OH^-} = 10^{14.58} 10^{-3,430/T} \qquad \text{(Eq. 11)}$$

$$k'_{Ac} = 10^{4.262} 10^{-1,674/T} \qquad \text{(Eq. 12)}$$

$$[AA]_s = 10^{3.845} 10^{-2,445/T} \qquad \text{(Eq. 13)}$$

where the k_i values are corrected in these equations to 0% dioxane as based on the comparison of 0% and 10% dioxane values at 25° and the temperature dependence of the k_i in 10% dioxane (8). Equations 10 to 13 are given in the nonlogarithmic form; $k_i = 10^{\log P} 10^{-S/T}$ of the Arrhenius equation, $\log k_i = \log P - S/T$, and T is the absolute temperature, where all k_i are in units of seconds.

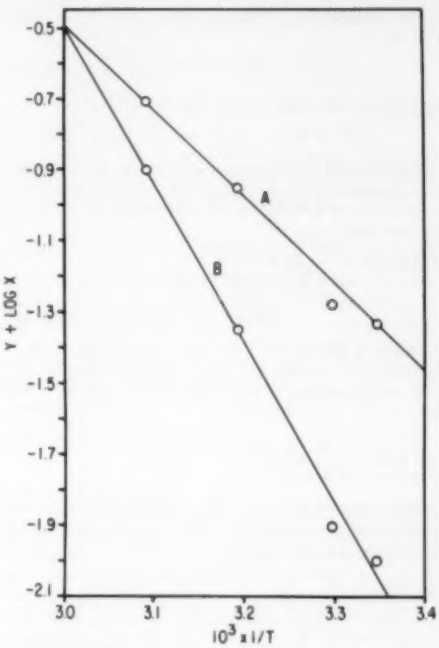


Fig. 5.—Temperature dependence of aspirin anhydride solubility, $[AA]_s$ in $M/1$, and rate of anhydride hydrolysis, $-d[AA]/dt$ in $M/1$/sec., in 0.1 N HCl. Curve A, y = 3 and x = $[AA]_s$. Curve B, y = 5 and x = $-d[AA]/dt = -k[AA]_s$.

The rate of appearance of salicylic acid, [sal], is first order on aspirin, [asp], until the solution is saturated with aspirin (9, 10):

$$d[sal]/dt = k_{asp}[asp] \qquad \text{(Eq. 14)}$$

where,

$$k_{asp} = k_1[H^+]/(1 + K'_a/[H^+]) + (k_4[H^+] + k_5 C_{H_2O} + k_6[OH^-])/(1 + [H^+]/K'_a) \qquad \text{(Eq. 15)}$$

$$k_1 = 10^{7.78} 10^{-3,690/T} \qquad \text{(Eq. 16)}$$

$$k_4 = 10^{9.05} 10^{-3,690/T} \qquad \text{(Eq. 17)}$$

$$k_5 C_{H_2O} = 10^{7.45} 10^{-2,940/T} \qquad \text{(Eq. 18)}$$

$$k_6 = 10^{9.18} 10^{-2,730/T} \qquad \text{(Eq. 19)}$$

$$K'_a = 2.4 \times 10^{-4} \qquad \text{(Eq. 20)}$$

The appearance of salicylic acid from aspirin anhydride is the result of a sequence of a zero-order and first-order reaction where:

$$[asp] + [sal] = 2kt \qquad \text{(Eq. 21)}$$

Until all anhydride has disappeared, the rate of accumulation of aspirin is equal to the difference in its rate of formation from anhydride by Eqs. 5 and 6 and its decomposition into salicylic acid by Eq. 14 so that

$$d[asp]/dt = -d[AA]/dt - d[sal]/dt \qquad \text{(Eq. 22)}$$

On integration, realizing that [asp] = 0 when $t = 0$,

$$[asp] = 2k(1 - e^{-k_{asp}t})/k_{asp} \quad \text{(Eq. 23)}$$

so that on the basis of Eqs. 21 and 23

$$[sal] = 2kt - 2k(1 - e^{-k_{asp}t})/k_{asp} \quad \text{(Eq. 24)}$$

Application of these equations at the physiological temperature of 37.5° gives the rate of anhydride hydrolysis as a function of pH from Eqs. 5, 9, 10, 11, and 13;

$$d[AA]/dt = [3.51 \times 10^{-2} + 3.4 \times 10^{3}[OH^-]]9.38 \times 10^{-5} = 32.9 \times 10^{-6} + 0.32[OH^-] \text{ in } M/\text{L./sec.} \quad \text{(Eq. 25)}$$

Below a pH of 6.0, the hydroxyl ion catalysis is insignificant so that the appearance of salicylic acid and aspirin on the basis of Eqs. 6, 21, and 25 would be

$$[asp] + [sal] = 2.37 \times 10^{-3}t \quad \text{(Eq. 26)}$$

where t is in hours.

The rate of salicylic acid appearance from the hydrolysis of aspirin at 37.5° may be calculated from Eqs. 14–20 where:

$$k_{asp}(sec^{-1}) = \underbrace{7.15 \times 10^{-5}[H^+]/(1 + 2.4 \times 10^{-4}/[H^+])}_{a} + \underbrace{(3.06 \times 10^{-3}[H^+]}_{b} + \underbrace{1.21 \times 10^{-5}}_{c} + \underbrace{0.24\ [OH^-]}_{d})/(1 + 4.17 \times 10^{3}[H^+]) \quad \text{(Eq. 27)}$$

The items in Eq. 27 have no significant contribution under the following pH conditions: *a* above 3.0, *b* above 4.0, *c* below 1.0 and above 11.0, and *d* below 8.0. Obviously, item *d* is of no physiological consequence.

At a pH of 1.0, the probable maximum acidity of the stomach, the salicylic acid concentration from a saturated solution of aspirin anhydride in excess as a function of time can be derived from Eq. 24 where t is in hours.

$$[sal] = 2.37 \times 10^{-3}t - 0.0834(1 - e^{-0.0264t}) \quad \text{(Eq. 28)}$$

Salicylic acid appearance from a saturated solution of aspirin in excess as a function of time at 37.5° may be derived from the knowledge of the k_{asp} values of Eq. 27 and the solubility of aspirin at 37.5° where $[asp]_s = 3.16 \times 10^{-2}$

$$[sal] = k_{asp}t \quad \text{(Eq. 29)}$$

where t is in hours and $k_{asp} = 8.98 \times 10^{-4}$ M/L./hr. at pH 1.0.

A comparison of salicylic acid production from saturated solutions of aspirin and aspirin anhydride at pH values of 0.5 and 1.0 over twenty hours is given in Fig. 6 as based on Eqs. 28 and 29. Similar relationships between salicylic acid concentrations from these sources will hold for all pH values.

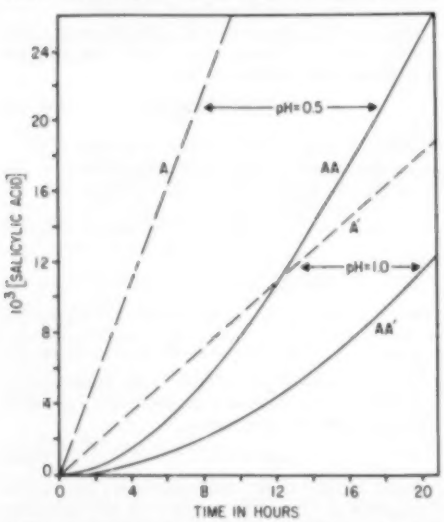


Fig. 6.—Comparison of salicylic acid production from saturated solutions of aspirin (curves A, A′) and aspirin anhydride (curves AA, AA′) at pH 0.5 (curves A, AA) and pH 1.0 (curves A′, AA′) at 37.5°.

DISCUSSION

Advantages of Anhydride Over Aspirin Relative to Gastric Irritation.—The prime disadvantages of aspirin therapy, those of digestive upset and gastric mucosal irritation, are well known. The literature (12) has provided strong evidence that aspirin is a gastric mucosal irritant.

As developed in the introduction, this irritation may be due to (*a*) salicylic acid production, (*b*) the natural acidity of aspirin, or (*c*) the adhesion of undissolved acidic aspirin to the gastric mucosa. A nonacidic "aspirin" would be ideal in that the rational causes of these disadvantageous phenomena would not be present. Aspirin anhydride fills the bill in that (*a*) its salicylic acid production under the pH conditions of the stomach (13) or the intestine is negligible compared to that from aspirin (see Fig. 6), (*b*) aspirin anhydride is nonacidic, and (*c*) aspirin anhydride has to dissolve to give aspirin, which when in solution may be almost instantaneously absorbed into the blood (11) so that the adhesion of irritating aspirin to the gastric mucosa cannot readily occur.

Advantages of Anhydride Over Aspirin Relative to Intestinal Absorption and Prolonged Action.—Feinblatt and Ferguson (14) have shown that within four minutes after the disintegration of a capsule in a fasted stomach, the capsule contents are in the process of leaving the stomach. Edwards (11) has measured the dissolution of aspirin, with agitation, of the 500-cc. covering liquid and has shown that, under these excellent solution-mixing conditions, only 0.6 of the aspirin in a 5-gr. tablet can be dissolved in three minutes at pH values of 1 to 2. This clearly indicates that with the less ideal agitation afforded by the empty or resting stomach, even less of the aspirin will be solubilized in the stomach, that a large fraction of the aspirin will find its way into the upper intestine prior to

solution, and even more than this fraction will enter the duodenum prior to absorption into the blood.

Edwards (11) has concluded that the absorption process of aspirin is extremely rapid and could be regarded as substantially complete within a few minutes of the aspirin dissolving; and that for aspirin taken in tablet form the rate-controlling process, for analgetic action resulting from blood concentration, is the dissolution within the stomach and intestine. The assumption was made that transfer rate was diffusion controlled rather than equilibrated with the blood on the blood side of the lipoid barrier. This may not be valid, since aspirin maxima in the blood are reached two hours after ingestion (15). Edwards considered the general case of all aspirin being able to diffuse which according to the Brodie, Hogben, Shore studies (16) is not so; only the undissociated aspirin may diffuse through the lipoid barrier.

It can be shown (Appendix, case C) that the general expression for the calculation of the concentration of an ionizable material in the blood, C_2, at time t is:

$$\log C_0 \Big/ \left\{ C_0 - C_2 \left[\left(\frac{1-\alpha_2}{1-\alpha_1} \right) + V/v \right] \right\} = 0.434\, DAt[[1-\alpha_2]v + [1-\alpha_1]V]/hvV \quad \text{(Eq. 30)}$$

where C_0 is the concentration in the gastrointestinal tract in a volume, v, where the material has an α_1 degree of dissociation; where the volume of the blood, V, is in instantaneous equilibrium and the degree of dissociation therein is α_2; where D is the diffusion constant, and h is the thickness, and A the effective area of the epithelial layer. On use of Edwards' parameters the Eq. 30 becomes for two 5-gr. tablets dispersed in the upper intestine or jejunum in a volume of $v = 500$ cc.

$$\log 1.3 / \left\{ 1.3 - C_2 \left[\left(\frac{1-\alpha_2}{1-\alpha_1} \right) + 6 \right] \right\} = 4.17 \times 10^{-3} t \ \{(1-\alpha_2)0.5 + (1-\alpha_1)3\} \quad \text{(Eq. 31)}$$

where t is in seconds.

Two probable conditions to consider are pH 6 or 7 for the intestine in equilibrium with material at pH 7 of the blood. The degrees of dissociation, α_1 and α_2, may be calculated at these pH values for aspirin of a $pK'_a = 3.8$ by

$$\alpha = \text{antilog}\,(\text{pH} - \text{pK}'_a)/[1 + \text{antilog}\,(\text{pH} - \text{pK}'_a)] \quad \text{(Eq. 32)}$$

so that Eq. 31 reduces to the form

$$\log [1.3/(1.3 - qc_2)] = rt \quad \text{(Eq. 33)}$$

where t is in minutes and if the intestinal contents are at pH 6.0, $1-\alpha_1 = 6.2 \times 10^{-3}$, $q = 6.1$, $r = 4.73 \times 10^{-3}$; and if the intestinal contents are at pH 7.0, $1-\alpha_1 = 6.2 \times 10^{-4}$, $q = 7$, and $r = 5.21 \times 10^{-4}$.

The aspirin content of the blood, VC_2, for pH 6 and 7 of the intestine, as a function of time is shown in Fig. 7 for one and two 5-gr. tablets in 500 cc. of intestinal volume.

The concentration of total salicylate (aspirin and aspirin anhydride) in the blood for a saturated solution of aspirin anhydride, $C_s = 0.03211$ Gm./L. at 37.5°, in an intestinal volume, $v = 500$ cc., is w/V where w may be given by (see Appendix, case B):

$$w = \frac{VC_sDA}{[Vhk + DA]} \left\{ kt + \frac{DA}{[Vhk + DA]} (1 - e^{-[k + DA/Vh]t}) \right\} \quad \text{(Eq. 34)}$$

and where $k = 3.85 \times 10^{-3}$ sec.$^{-1}$ is the first-order rate constant for the hydrolysis of aspirin anhydride.

On use of Edwards' parameters (11) Eq. 34 becomes

$$w(\text{Gm.}) = 0.0123t + 0.0296(1 - e^{-0.5194t}) \quad \text{(Eq. 35)}$$

where t is in minutes. The contribution of aspirin anhydride to the total salicylate concentration in the blood will not exceed 1.78×10^{-4} Gm./cc. since it is so readily hydrolyzed in solution to aspirin. The rate of diffusion of aspirin anhydride through the intestinal wall is constant for this steady state condition reached within ten minutes and is (see Appendix, case C),

$$w\,(\text{Gm.}) = 0.0123t \quad \text{(Eq. 36)}$$

where t is in minutes.

The calculated values plotted in Fig. 7 clearly show that aspirin anhydride in the intestine provides aspirin to the blood at a much faster rate than aspirin at concentrations given by two or one 5-gr. tablets per 500 cc. of intestinal volume, at intestinal pH values of 6 or 7.[1] The total acetylsalicylate appearance from aspirin anhydride is independent of the intestinal pH, volume, or number of tablets in the intestine; its rate is a constant as long as sufficient anhydride is available to exceed the low solubility. The pH of the intestine ranges from 6 to 7 and thus it can be concluded that the acetylsalicylate appearance rate from aspirin is at least intermediate between curves B and D of Fig. 7.

Table III gives salicylate blood levels at several time intervals on the basis of different intestinal conditions. Metabolic elimination was assumed to be inoperative from both the gastrointestinal tract and the blood in the calculation of these values.

Figure 7 and Table III may not give the true

TABLE III.—ACETYLSALICYLATE CONTENT OF pH 7 BLOOD IN GM./L. AS A FUNCTION OF TIME FROM ASPIRIN AND ASPIRIN ANHYDRIDE IN THE INTESTINE

	Gr./500 cc. Intestinal Volume			
	Aspirin			Aspirin Anhydride
	10	5	10	Any[a]
Time, Min.	Intestinal pH			
	6	6	7	Any
5	0.0113	0.0057	..	0.030
10	0.0220	0.0110	0.0022	0.051
60	0.102	0.051	0.013	0.256
100	0.141	0.071	0.021	0.420
1,000	0.212[b]	0.106[b]	0.130	4.11
10,000	..	..	0.185[b]	..

[a] Total transfer of 5 gr. of aspirin anhydride is accomplished within twenty-four minutes; of 10 gr. within fifty minutes. Hydrolysis to aspirin in intestine and metabolic elimination are ignored.

[b] Maximum blood concentration achievable due to the necessary equilibrium between intestinal contents and blood. Metabolic elimination is ignored.

[1] Dr. W. L. Miller, Jr., of these laboratories has confirmed this prediction of higher aspirin blood levels from aspirin anhydride oral administration over aspirin oral administration in humans and will publish his findings shortly.

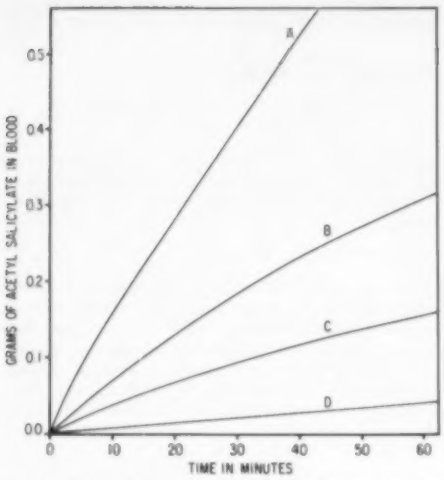


Fig. 7.—Appearance of acetylsalicylate in the blood from aspirin and its anhydride at several intestinal pH values and concentrations. Curve A is for aspirin anhydride at any pH and at any dosage in excess of saturation. Curve B is for two 5-gr. tablets of aspirin in 500 cc. of intestinal fluid at pH of 6 whereas curve C is for one such tablet. Curve D is for two 5-gr. tablets of aspirin in 500 cc. at an intestinal pH of 7.

values of blood concentration because of the probable invalidity of the assumption that the blood is in instantaneous equilibrium with the material in the lipoid barrier. However, the relative values should be valid for comparison of total acetylsalicylate blood levels from aspirin and aspirin anhydride. However, the aspirin maxima in the blood from aspirin ingestion calculated on these bases are reached in about two hours after ingestion. This fact is consistent with experimental evidence, with the postulated importance of intestinal absorption, and the pH dependence of aspirin absorption in the intestine. It is inconsistent with the results of Edwards' premise (11) of pH independent absorption in the gastrointestinal tract, that aspirin absorption into the blood is virtually instantaneous.

SUMMARY AND CONCLUSIONS

1. Aspirin anhydride, as well as being a nonacidic drug, fulfills other rational criteria for a superior aspirin dosage form. From the physicochemical evidence: (*a*) Aspirin blood levels from aspirin anhydride will be higher than from aspirin ingestion due to the latter's relatively slow, pH-dependent absorption from the intestine. This should be most pronounced at low stomach retention times, e. g., with fasted individuals. (*b*) The salicylic acid content in the gastrointestinal fluids and in the blood will be higher at any time from aspirin ingestion than from the ingestion of aspirin anhydride. This is due to the routes of hydrolysis of these two substances.

2. The heterogeneous hydrolysis of aspirin anhydride has been studied at several temperatures and its aqueous solubilities have been determined. Complete equations have been presented for the hydrolysis of this material at the physiological temperatures and pH values.

3. Procedures have been developed to determine aspirin, aspirin anhydride, and salicylic acid spectrophotometrically.

4. The solubility equilibrium procedure presented will permit the estimation of aspirin in aspirin anhydride without the need of a primary standard. The procedure has been applied.

APPENDIX

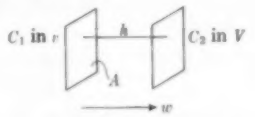
Derivation of Equations for Calculation of Diffusion of Amounts of Various Aspirin Derivatives from the Intestine into the Blood

Edwards (11) has assigned values to the necessary parameters to calculate the concentration of aspirin in the blood on diffusion through the intestinal wall. He concluded that the rate-controlling process for analgetic action in the blood is the dissolution of aspirin within the stomach and intestine. However, the basic premise of his calculations and conclusions was that all aspirin in the intestine diffuses into the blood stream. This is not believed to be the case; only undissociated aspirin may be presumed to diffuse through the lipoid barrier (16).

The General Case.—Fick's law of diffusion (17) states that the quantity dw diffusing across A cm.^{2} of area in time dt with a concentration gradient of $(C'_2 - C'_1)/h$ may be expressed mathematically by

$$dw/dt = -AD(C'_2 - C'_1)/h \qquad \text{(Eq. 1)}$$

The following figure shows that w Gm. of material can be transported across h cm. of intestinal wall with a total area of A cm.^{2} and a diffusion constant of D cm.^{2}/sec. when the total concentration of the material is C_1 Gm./cc. in a volume, v, in the intestine, and where the concentration is C_2 Gm./cc. in a volume V of the blood.



The material balance in the intestine and blood can be expressed by

$$vC_0 = vC_1 + VC_2 \qquad \text{(Eq. 2)}$$

where C_0 is the original concentration of the material in the intestine at time $t = 0$, prior to diffusion.

All calculations are to be based on Edwards' parameters, i. e., two 5-gr. tablets are dispersed in the upper intestine or jejunum in a volume of $v = 500$ cc., giving an apparent $C_0 = 1.3 \times 10^{-3}$ Gm./cc., where $D = 1.2 \times 10^{-5}$ cm.^{2}/sec., $A = 3 \times 10^2$ cm.^{2}, $V = 3 \times 10^3$ cc., and $h = 2.5 \times 10^{-3}$ cm., and homogeneous dispersion in the blood is assumed.

Case A. Diffusion Through the Intestinal Wall of Undissociated Ionizable Materials.—The amount

diffused through the intestinal wall at a time t is

$$w = C_2V \quad \text{(Eq. 3)}$$

but the actual concentration gradient is set up by undissociated molecules so that if α_1 is the degree of dissociation at the pH of the intestine and α_2 at the pH of the blood, then the effective concentration of diffusable materials is $(1 - \alpha_1)C_1$ so that the concentration gradient in this case is actually

$$(C'_2 - C'_1)/h = [(1 - \alpha_2)C_2 - (1 - \alpha_1)C_1]/h \quad \text{(Eq. 4)}$$

Substitutions of eqs. 3 and 4 into 1 and using the value of C_2 as calculated from Eq. 2, and rearranging in integrable form, we have

$$\int_0^{C_2} \frac{dC_2}{[1 - \alpha_1]vC_0 - C_2[[1 - \alpha_2]v + [1 - \alpha_1]V]} = \int_0^t \frac{DA}{hvV}\,dt \quad \text{(Eq. 5)}$$

which integrates to

$$\ln C_0 \Big/ \left\{C_0 - C_2\left[\left(\frac{1 - \alpha_2}{1 - \alpha_1}\right) + V/v\right]\right\} = DAt[[1 - \alpha_2]v + [1 - \alpha_1]V]/hvV \quad \text{(Eq. 6)}$$

Substitution of the appropriate numerical values cited permits the simplification of Eq. 6 to

$$\log 1.3 \Big/ \{1.3 - C_2[(1 - \alpha_2)/(1 - \alpha_1) + 6]\} = 4.17 \times 10^{-2}t[(1 - \alpha_2)0.5 + (1 - \alpha_1)3] \quad \text{(Eq. 7)}$$

Case B. Diffusion Through the Intestinal Wall of Material at Constant Soluble Concentration, C_s, in the Intestinal Contents, Which Is Transformed at a Finite Rate to Nondiffusible Material in the Blood.—If the concentration of diffusible material in the intestine is a constant, i. e., $C'_1 = C_s = 3.2 \times 10^{-5}$ Gm./cc. (solubility of AA at 37.5°) as when the amount available exceeds its solubility, and if this material is readily transformed in the blood at concentration C_2 to a nondiffusible charged material of concentration C_3 by first-order kinetics, then

$$dC_3/dt = kC_2 \quad \text{(Eq. 8)}$$

where $k = 3.85 \times 10^{-3}$ sec.$^{-1}$ at 37.5° and the concentration gradient in this case is

$$(C'_2 - C'_1)/h = (C_2 - C_s)/h \quad \text{(Eq. 9)}$$

The amount diffused through the intestinal wall at a time t is

$$w = [C_2 + C_3]V \quad \text{(Eq. 10)}$$

so that on substitution of Eqs. 9 and 10 into 1

$$dw/dt = Vd[C_2 + C_3]/dt = -DA(C_2 - C_s)/h \quad \text{(Eq. 11)}$$

When Eq. 8 is used, rearrangement results in the linear equation

$$dC_2/dt + [k + (DA)/(Vh)]C_2 = (DAC_s)/(Vh) \quad \text{(Eq. 12)}$$

The integrating factor is $e^{\int [k + (DA)/(Vh)]dt} = e^{[k + (DA)/(Vh)]t}$ so that Eq. 12 may be integrated between the limits 0 and C_2, 0 and t, rearranged and simplified to

$$C_2 = \frac{DAC_s}{[Vhk + DA]}\{1 - e^{-[k + (DA)/(Vh)]t}\} \quad \text{(Eq. 13)}$$

Substitution of the appropriate numerical values cited permits the simplification of Eq. 13 to

$$C_2 = \frac{1.2C_s}{(250k + 1.2)}\{1 - e^{-[k + 4.8 \times 10^{-3}]t}\} = 1.78 \times 10^{-5}\{1 - e^{-4.65 \times 10^{-3}t}\} \quad \text{(Eq. 14)}$$

To calculate the total amount, w, of material that passed through the membrane, Eq. 13 or 14 may be substituted into Eq. 11 and integrated between the limits 0 and w, 0 and t,

$$w = \frac{VC_sDA}{[Vhk + DA]}\left\{kt + \frac{DA}{[Vhk + DA]}(1 - e^{-[k + DA/Vh]t})\right\} = \frac{VDA}{[Vhk + DA]}[C_skt + C_3] \quad \text{(Eq. 15)}$$

Substitution of the appropriate numerical values cited permits the simplification of Eq. 15 to

$$w = \frac{108C_s}{7.5k + 3.6 \times 10^{-2}}\left\{kt + \frac{3.6 \times 10^{-2}}{7.5k + 3.6 \times 10^{-2}}(1 - e^{-[k + 4.8 \times 10^{-3}]t})\right\} = 20.5 \times 10^{-5}t + 2.96 \times 10^{-2}(1 - e^{-4.65 \times 10^{-3}t}) \quad \text{(Eq. 16)}$$

Case C. The Steady State of Case B.—When $t > 600$ sec., the second factor in Eq. 14 approaches unity so that the steady state concentration of material not transformed to C_3 is

$$C_2 = 1.2C_s/(250k + 1.2) = 1.78 \times 10^{-5} \text{ Gm./cc., } t > 600 \quad \text{(Eq. 17)}$$

Thus, the steady state diffusion through the intestinal wall from Eq. 11 becomes

$$dw/dt = 20.4 \times 10^{-5} \text{ Gm./sec.} \quad \text{(Eq. 18)}$$

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Prediction of Stability in Pharmaceutical Preparations VI*

Stability, Products, and Mechanism in the Pyrolytic Degradation of Aspirin Anhydride

By EDWARD R. GARRETT, EDWARD L. SCHUMANN, and MARVIN F. GROSTIC

The effects of crystallizing solvent and moisture on the pyrolytic degradation of aspirin anhydride were studied by infrared spectroscopy. The unexpected effects of "soft glass"-catalyzed surface decomposition were observed. Micronization, drying, and avoidance of contact with alkali increased the thermal stability. A mechanism of acyl transfer in the formed melt adequately explains the products identified by isolation and infrared studies.

THE APPLICATION OF prediction stability to pharmaceuticals that degrade by solvolytic processes is generally quite straightforward. The degradation may usually be assigned to pseudo zero- or first-order kinetics and the classical laws of physicochemical kinetics are quantitatively applicable (1–5). When solid materials, as powders or in admixtures of powders, are studied for stability prediction, the manner and rates of degradation may be more complex. Appropriate treatment of the data may not give as elegant a quantitative prediction as for materials in solution but can give good estimates and proper ranking of various alternate preparations.

Properly designed, frequently assayed accelerated stability studies may permit the evaluation of assay methods and of artifacts giving erroneous assay.

There is physical chemical evidence that aspirin anhydride is a superior form for the oral administration of aspirin (6, 7). Since logical dosage forms are as tablets or powders, the prediction of stability in these forms is of interest. This paper reports on these stability studies and the products and mechanism in the pyrolytic degradation of aspirin anhydride.

EXPERIMENTAL

Initial Design for Study of Pyrolytic Stability of Aspirin Anhydride in Soft Glass.—Twenty-five milligrams of aspirin anhydride were weighed out in 15 × 45-mm. glass vials (Kimble Opticlear) with press fit polyethylene caps. Twenty of each lot were placed in large sealed ointment-type jars each of which was submerged beneath the oil of 70, 60, 50, and 40° constant temperature baths. Ten vials of each lot were left at room temperature and in a refrigerator at 4°.

The material was assayed by dissolution in 5 cc. $CHCl_3$ and evaluation of the resultant infrared spectra in the 1,600–1,850 cm.$^{-1}$ region. The spectral carbonyl region of possible hydrolytic or other end products of aspirin anhydride degradation, i. e., acetic anhydride, aspirin (asp), and salicylic acid (sal) is given in Fig. 1. Estimates of the nonpyrolyzed purity of aspirin anhydride (AA) have been made from a 3-component assay at 1,782 cm.$^{-1}$, 1,700 cm.$^{-1}$, and 1,668 cm.$^{-1}$ using supposedly pure components as standards.

The first three lots studied in this manner are given in Table I.

Pyrolytic Degradation of Aspirin Anhydride in Soft-Glass Vials.—A typical example of the change of carbonyl spectra with time on the pyrolytic degradation of aspirin anhydride is given in Fig. 2, i.e., lot I at 60°. Three pertinent wavelengths for observation are obviously at 1,787.5 (where absorbance decreases on pyrolysis), 1,737.5, and 1,700 cm.$^{-1}$ (where absorbances increase on pyrolysis). An apparent isobestic point can be observed at 1,755 cm.$^{-1}$.

TABLE I.—INITIAL PYROLYTIC STUDIES ON ASPIRIN ANHYDRIDE

Lot	Comments	Recrystallization Solvent	H_2O[a], %
I	Original I. R. assay 100.0%, negligible asp and sal	Isopropyl alcohol-acetone	1.21
II	Salvage material from many lots. I. R. assay 94.6% AA, 0.6% asp, negligible sal	Isopropyl alcohol acetone	No weight loss
III	Purity >99% by solubility analysis in ethylacetate-*n*-heptane, 1:10, 100.1% AA by I. R.	Diethyl ether	No weight loss

[a] By weight loss at 0.01 mm. Hg, twenty-five hours at room temperature.

* Received May 21, 1959, from The Research Division, The Upjohn Co., Kalamazoo, Mich.
The prior paper in this series is given in reference (1).
The authors are greatly indebted to Mrs. Lillian G. Snyder and Mr. George E. Bronson for excellent technical assistance and to Mr. W. A. Struck and his associates in our Analytical Laboratory for the analytical data.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

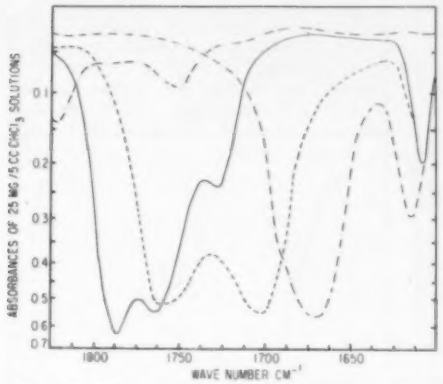


Fig. 1.—The carbonyl region infrared spectra of aspirin anhydride and hydrolytic products of amounts equivalent to 25 mg. aspirin anhydride in 5 cc. $CHCl_3$: aspirin anhydride ——, aspirin - - - -, salicylic acid - · - · -, acetic anhydride- · · - · · -.

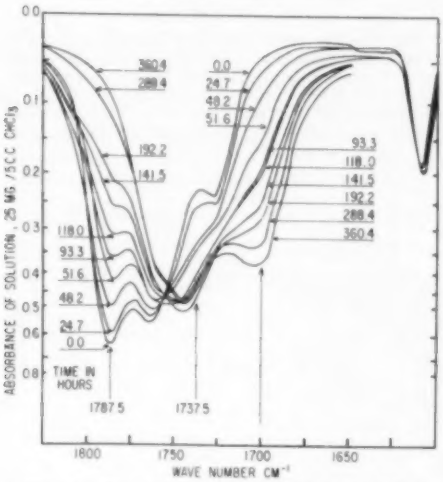


Fig. 2.—The carbonyl region infrared spectra of aspirin anhydride (lot I) degraded at 60° in "soft" glass vials. The amount of aspirin anhydride was 25 mg.; the spectra were run in 5 cc. of $CHCl_3$.

Comparison of Fig. 2 with Fig. 1 definitely shows that salicylic acid is not a product of the pyrolytic degradation since there is no appearance of a 1,668 cm.$^{-1}$ salicylic acid band in Fig. 2.

Although the aspirin spectra of Fig. 1 may account for part of the end product at three hundred and sixty hours, it obviously does not account for the whole since the absorbance at 1,700 cm.$^{-1}$ is much less than the absorbance at 1,750 cm.$^{-1}$ in Fig. 2. These absorbances are almost equivalent for aspirin in Fig. 1.

The three hundred and sixty-hour spectra (Fig. 2) subsequently changed little with time. The nature of any change was a decrease in absorbances at all frequencies, indicating volatilization or loss of end product rather than any subsequent transformation.

Typical plots of absorbance *vs.* time at 1,787.5 cm.$^{-1}$ are given in Fig. 3 for the several temperatures of degradation. The three lots, I, II, and III did not show any highly significant differences in 70 and 60° degradation and the plot of only one, lot I, is given in Fig. 3. The curves did not fit zero-order or first-order type plots in their entirety. The best characterization would be an initial linear loss of absorbance with a subsequent logarithmic or first-order type loss.

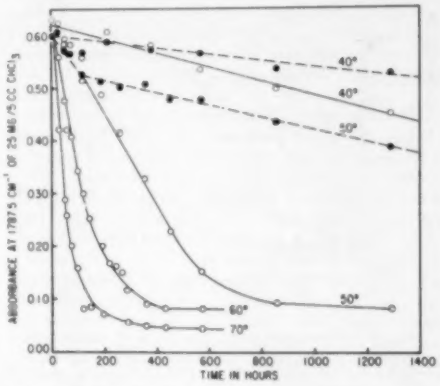


Fig. 3.—Pyrolytic degradation of typical aspirin anhydride lots in "soft" glass as measured by the absorbance of 25 mg. in 5 cc. $CHCl_3$ against time at 1,787.5 cm.$^{-1}$; lot I ○, lot II ●.

However, at 50 and 40° a distinct difference among lots in loss of absorbance with time can be observed. Although lots II and III demonstrated similar degradation rates at these temperatures (only plots for lot II are shown in Fig. 3), lot I degraded at a dramatically greater speed.

Inspection of the rate of absorbance increases at 1,700 and 1,737.5 cm.$^{-1}$ as given in Fig. 4 showed similar phenomena. All three lots similarly degraded at 70 and 60° and lots II and III degraded similarly at the other temperatures. However, lot I degraded at a much faster rate than the other two. This is most clearly shown in the 50° studies at these wave numbers.

Visual observation of the degradation allowed the following conclusions. The appearance of liquid occurred rather quickly and within twenty-four hours at 70° complete liquefaction had set in. At the lower temperatures, change in carbonyl spectra could be qualitatively correlated with the degree of liquefaction; lot I being completely liquefied much before the others. With decomposition, acetic acid or acetic anhydride could be smelled.

In an attempt to manufacture a large amount of pyrolytic end product 10 Gm. of lot I was sealed within a Carius tube and submerged in the 70° bath.[1] Inspection of the curves (Fig. 3) had indicated that complete transformation should have occurred within seven days. The material was completely liquefied within two to three days. However, an infrared spectrum of the resultant carbonyl region was not the same as that for three hundred and sixty hours at 60° in Fig. 2 or four hundred and thirty-two

[1] This work was performed by Dr. E. C. Olson of our laboratories.

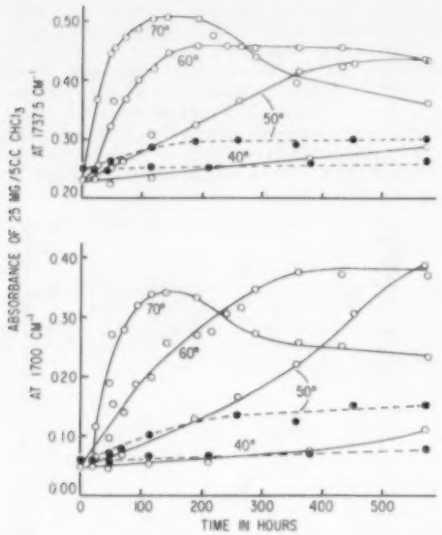


Fig. 4.—Pyrolytic degradation of typical aspirin anhydride lots in "soft" glass as measured by the absorbance of 25 mg. in 5 cc. $CHCl_3$ against time at 1,700 and 1,737.5 cm.$^{-1}$; lot I ○; lot II ●.

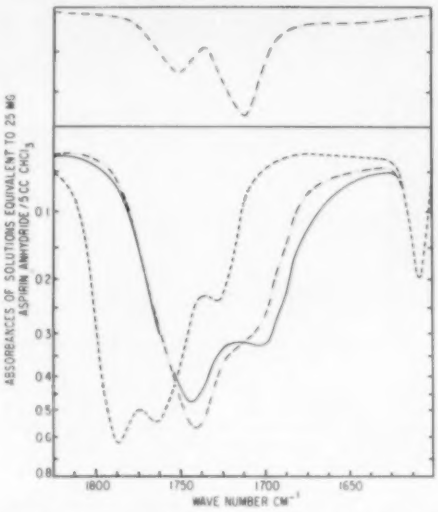


Fig. 5.—The carbonyl region infrared spectra of aspirin anhydride and possible pyrolytic end products of amounts equivalent to 25 mg. aspirin anhydride in 5 cc. $CHCl_3$: aspirin anhydride - - - -; end product of 70° pyrolysis at four hundred and thirty-two hours ——; acetylsalicoylsalicylic acid - - - - - - - -; acetic acid - - - - -.

hours at 70° in Fig. 5. The probably erroneous initial conclusion was that it was impure aspirin anhydride; the carbonyl region spectra were similar to that given for the anhydride of acetylsalicoylsalicylic acid in Fig. 6.

In addition, 10 Gm. of lot I were heated in a Pyrex tube at 70° for one week with a slow stream of air passing through the material. Volatile materials were trapped and identified as water and acetic acid.[1] The material did not liquefy. Infrared identified it as slightly decomposed aspirin anhydride.

Comparison of the Pyroly' Stability of Aspirin Anhydride in "Soft" and ard" Glass Containers.—As previously, 25 mg. of aspirin anhydride was weighed out in the glass vials, i. e., "soft" glass. Similarly, the material was weighed out in Pyrex or so-called "hard" glass. These latter were sealed with corks wrapped in aluminum foil.

A dramatic difference was observed in the pyrolytic degradation of material in these two types of containers. This is graphically shown in Fig. 7 where the aspirin anhydride from the same lot pyrolyzed in "soft" glass showed large changes in carbonyl spectra at 1,787.5 cm.$^{-1}$ compared to those pyrolyzed in "hard" glass at both 70 and 50°.

Three lots (see Table II) of anydride showed no difference among themselves when pyrolyzed in "hard" glass at 50 and 70°. However, in "soft" glass, anhydride recrystallized from absolute ethanol showed a definitely worse stability (lot V) over material finally recrystallized from Skelly B-ether (lot IV) and Skelly B-ethyl acetate (lot VI). These facts indicate that ethanol of solvation is difficult to remove from the anhydride and may not be the recrystallization solvent of choice even though the nicest appearing and largest crystals resulted from this solvent.

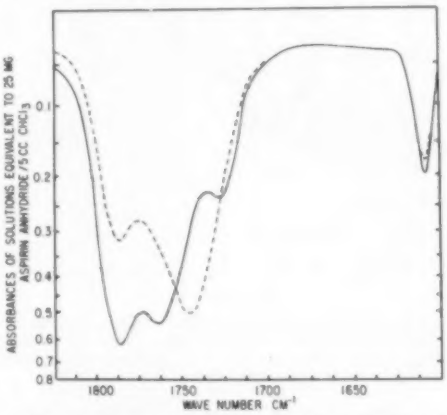


Fig. 6.—The carbonyl region infrared spectra of aspirin anhydride and a possible pyrolytic intermediate, equivalent to 25 mg. aspirin anhydride in 5 cc. $CHCl_3$: aspirin anhydride ——, 2,2′-bis(2-acetoxybenzoyloxy)benzoic anhydride - - - -.

To check the effect of the plastic cap, finely ground plastic caps were placed in contact with anhydride in the "hard" glass containers. No difference in degradation rate from the material alone in "hard" glass could be observed.

Comparison of Pyrolytic Stability of Micronized, Nonmicronized, and Impure Aspirin Anhydride.—The pyrolytic stability of aspirin anhydride was studied in screw-cap polyethylene containers. The material was subjected to varying temperatures and infrared-spectrophotometrically analyzed as previ-

ously described. Properties of the anhydride lots studied are tabulated in Table III.

No great difference in pyrolytic degradation among lots VII, IX, and X was observed. The plots of lot VII, absorbance at 1,787.5 cm.$^{-1}$ *vs.* time for 70, 60, and 50° in Fig. 8 is characteristic of all three of these lots. From this it can be concluded that the two different solvent mixtures of crystallization, ethyl acetate-Skelly B and acetone-isopropyl alcohol, are indistinguishable in their effects on the pyrolytic degradation of thoroughly dried aspirin anhydride. It is also inidcated that an admixture, containing up to 3% aspirin, has no enhanced pyrolytic degradation. However, it must be realized that this is not the same case as aspirin impurity molecularly dispersed throughout aspirin anhydride.

A synthetic admixture of anhydride with salicylic acid (i. e., 4.8%) does have significantly greater pyrolytic degradation rates than aspirin anhydride itself. The increased rates of degradation of lot XI over lot VII are clearly demonstrated in Fig. 8 at all three temperatures.

Micronized aspirin anhydride with subsequent thorough drying demonstrated highly significant increases in pyrolytic stability. Lot VIII, which is lot VII micronized and redried, shows an astonishing increase in stability over lot VII at all temperatures when the pertinent I. R. absorbances are plotted against time, as in Fig. 8.

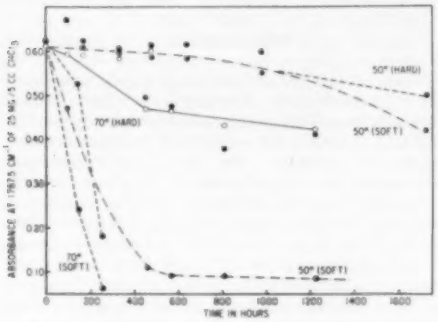


Fig. 7.—Comparative effects of Pyrex and "soft" glass containers on the pyrolytic degradation of aspirin anhydride as measured by the absorbance of 25 mg. in 5 cc. $CHCl_3$ against time at 1,787.5 cm.$^{-1}$. The solid lines are for "soft" glass containers and the dashed are for Pyrex: lot IV ○; lot V ◑; lot VI ●.

TABLE II.—CHARACTERIZATION OF LOTS USED IN COMPARISON OF "SOFT" AND "HARD" GLASS CONTAINERS ON PYROLYTIC STABILITY OF ASPIRIN ANHYDRIDE

Lot	M. p. Uncorr., °C.	I. R. Assay AA, %	I. R. Assay Aspirin, %	Recrystallization Solvents
IV	84–85	101.0	0.1	3× from ethyl acetate-Skelly B 2:3; 1× from absolute ether–Skelly B 2:1
V	83–85	100.8	0.2	2× from ethyl acetate-Skelly B; 2:3; 1× from absolute ethanol
VI	84–86	100.3	0.0	2× from ethylacetate-Sk lly B; 2:3; 1× from acetone-Skelly B; 1:1; 1× from ethylacetate-Skelly B; 1:1

Preparation of the Possible Products of Pyrolytic Degradation.—Six compounds were prepared for I. R. spectrophotometric comparison with thermally decomposed aspirin anhydride: *Acetylsalicoylsalicylic Acid, $C_{16}H_{12}O_6$, mol. wt. 300.3.*—This substance was prepared by the method of A. Einhorn

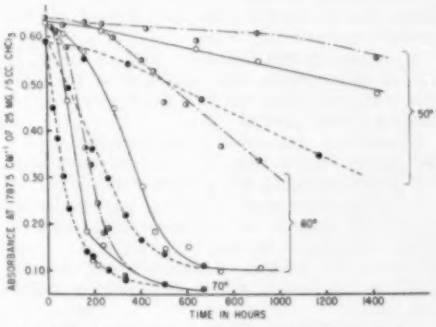


Fig. 8.—Comparison of the pyrolytic degradations of crystalline, micronized, and salicylic acid-contaminated aspirin anhydride in polyethylene bottles. The absorbance of 25 mg. in 5 cc. $CHCl_3$ was measured against time at 1,787.5 cm.$^{-1}$; lot VII, crystalline aspirin anhydride ○; lot VIII, micronized ◑; lot XI, aspirin anhydride plus 4.8% salicylic acid ●.

TABLE III.—CHARACTERIZATION OF LOTS USED IN COMPARISON OF MICRONIZATION, NONMICRONIZATION, AND ASPIRIN AND SALICYLIC ACID IN ADMIXTURES ON THE PYROLYTIC STABILITY OF ASPIRIN ANHYDRIDE

Lot	Recrystallization	Remarks	Assay by I. R.
VII and VIII	2× from ethyl acetate-Skelly B, 2:3	Dried overnight at room temperature at 2 mm. of Hg. then 24 hr. at 2 mm.; 0.02% wt. loss	99% AA 0% aspirin 0.1% salicylic acid
IX	Same as lot VII with a subsequent recrystallization 2× from acetone-isopropyl alcohol	Dried at 12 mm. for 24 hr., wt. loss 0.08%	101% AA 0.2% aspirin 0.1% salicylic acid
X	Synthetic batches derived from lot VII + salicylic acid + aspirin		96.6% AA 2.6% aspirin 0.3% salicylic acid
XI			95.1% AA 0.7% aspirin 4.8% salicylic acid

(8) who reported a melting point of 161–162°. Material obtained in this study melted at 163–165° (uncorr.) after recrystallization from benzene and 95% ethanol.

2,2′-Bis(2-acetoxybenzoyloxy)-benzoic Anhydride, $C_{32}H_{22}O_{11}$, mol. wt. 582.4.—A mixture of 9.0 Gm. (0.03 mole) of acetylsalicoylsalicylic acid in 15 ml. of methylene chloride and 2.4 Gm. (0.03 mole) of pyridine was stirred and cooled in an ice bath during the addition of a solution of 1.8 Gm. (0.015 mole) of $SOCl_2$ in 10 ml. of methylene chloride over a five-minute period. The mixture was maintained at 5–8° and stirred fifteen minutes longer, then poured over a mixture of 100 ml. of crushed ice and 15 ml. of 10% HCl and mixed thoroughly.

The methylene chloride layer was separated, dried over $MgSO_4$, and evaporated. The residue, which crystallized on standing, was recrystaliized twice from ethyl acetate-Skelly B (1:1) to give 5.7 Gm. (65%) of fine, white needles, melting point 142–144° (uncorr.).

Anal.—Calcd. for $C_{32}H_{22}O_{11}$: C, 65.98; H, 3.81. Found: C, 65.95; H, 3.83.

Mixed Anhydride of Aspirin and Acetic Acid, $C_{11}H_{10}O_5$, mol. wt. 222.2.—This compound and the mixed anhydride of acetylsalicoylsalicylic and acetic acids are very labile, decomposing at room temperature to give the expected symmetrical anhydrides. No satisfactory carbon-hydrogen analyses were obtained due to decomposition and loss of varying amounts of acetic anhydride.

A solution of 18.0 Gm. (0.1 mole) of aspirin in 600 ml. of anhydrous ether was treated, via a gas inlet tube, with excess ketene. The ketene was produced in a Hurd generator (9).

Solvent was removed at room temperature by a dry nitrogen stream and the residual colorless oil was stored at 0° until used for I. R. analysis.

Pyrolysis of the mixed anhydride was effected by heating the material on the steam bath under reduced pressure (*ca.* 40 mm. for 90 minutes). Trituration of the residual oil with 1:1 ethyl acetate-Skelly B and seeding gave a 46% yield of aspirin anhydride.

Mixed Anhydride of Acetylsalicoylsalicylic and Acetic Acids, $C_{18}H_{14}O_7$, mol. wt. 342.3.—A solution of 6.0 Gm. of acetylsalicoylsalicylic acid in 200 ml. of anhydrous acetone was treated with ketene as described above and worked up in the same way. The residual oil was stored at 0° until used.

Material which was allowed to stand at room temperature decomposed to give 2,2′-bis(2-acetoxybenzoyloxy)-benzoic anhydride.

Disalicylide and Trisalicylide.—These products were prepared by pyrolysis of aspirin at 300–350° at 10–15 mm., using the procedure of Baker, Ollis, and Zealley (10).

Infrared analysis showed no trace of these compounds in any of the decomposed aspirin anhydride samples examined.

Isolation of Acetylsalicoylsalicylic Acid from Decomposed Aspirin Anhydride.—Acetylsalicoylsalicylic acid was isolated from decomposed moist aspirin anhydride, lot I, pyrolyzed at 70° for three weeks in the presence of soft glass, in the following manner:

An ether solution of decomposed aspirin anhydride was extracted with 5% aqueous $NaHCO_3$ and the bicarbonate solution was acidified with acetic acid and extracted with ether. The ether solution was dried over $MgSO_4$ and then evaporated to give crude acetylsalicoylsalicylic acid which was almost identical to authentic material.

2,2′-Bis(2-acetoxybenzoyloxy)-benzoic anhydride could not be isolated from the complex of aspirin anhydride decomposition products.

DISCUSSION

Three aspects of these studies on aspirin anhydride are to be considered. The first is the effect of moisture, solvent, alkali, purity, and micronization on the stability of aspirin anhydride and the conditions for maximum stability. The second is a proposed mechanism of acyl exchange for the pyrolytic degradation of aspirin anhydride and its consistency with the observed changes in infrared spectra of the carbonyl region as compared to the infrared spectra of compounds that could result from acyl interchange. The third is the further evidence from isolation of degradation products and comparison of complete infrared spectra.

Stabilization of Aspirin Anhydride Against Pyrolytic Degradation.—It is apparent that water contamination increases the thermal instability of aspirin anhydride even in amounts as low as 1%. This is aptly demonstrated by the plots of Fig. 3 for lots I and II.

Material in contact with "soft," or high alkali content, glass has the greater pyrolytic instability. This is demonstrated by the curves of Fig. 7. Comparison of the curves of Fig. 8 for polyethylene with those of the "soft" glass containers of Fig. 6 also substantiates this point.

Contamination with salicylic acid adversely affects the pyrolytic stability as can be seen in Fig. 8.

It can be concluded from the above facts and other observations given in "Experimental" that the pyrolytic degradation of aspirin is initiated on the surface of the crystals, that it is catalyzed by the higher alkali content of contacted "soft" glass, that the surface-formed products lower the melting point, originally *ca.* 84°, to form a melt. The pyrolytic degradation proceeds at a faster rate within the melt.

When aspirin anhydride contains moisture (as in lot I) or is contaminated by solvent (as presumed in lot V), liquefaction and thus pyrolytic degradation is accelerated. Under these conditions and especially when alkali is present for catalysis, as in "soft" glass, no induction period may be readily apparent (Fig. 3). However, when the anhydride is thoroughly dried, of high purity, and not in surface contact with alkali, its pyrolytic stability is greatly enhanced, as with lots VII and VIII in Fig. 8; the induction period becomes obvious. Any material, such as salicylic acid, which significantly lowers the melting point and abets liquefaction will favor instability.

Removal of the initial products of pyrolytic degradation of aspirin anhydride, for example, acetic anhydride or acid, or unbound or released solvent as by a stream of heated air, inhibits liquefaction and stabilizes.

The increased stability of micronized anhydride over unmicronized (lot VIII over VII in Fig. 8) or of small crystals from ethyl acetate-Skelly B over the large crystals from ethanol crystallization (lot VI over

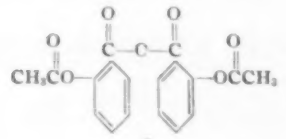
lot V in Fig. 7) are unexpected phenomena. This is especially so in the former case where lot VII showed negligible weight loss on drying (0.02%). A possible explanation is that large crystals are solvates or contain trapped solvent, that smaller or fractured crystals are more liable to lose this solvent of crystallization when subject to higher temperatures. Thus the melting points would not be lowered as much so that it is more difficult to produce the "melt" wherein pyrolytic degradation is accelerated.

Thus, to gain maximum stability to pyrolytic degradation, the aspirin anhydride should not be contaminated with salicylic acid, should be thoroughly dried, and micronization is highly desirable. Since contact with alkali or Bronsted-type bases may catalyze the decomposition, the absence of contacting alkaline surfaces should promote stabilization.

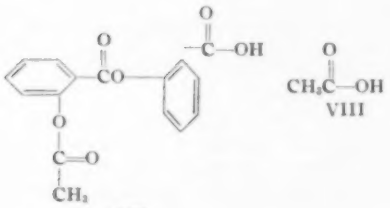
Removal of the products of pyrolysis, as by hot air stream, which could promote liquefaction and degradation will stabilize.

The studies on lot VII (Fig. 8), a dry pure material, show that for a 10° temperature decrease from 70 to 60°, the estimated linear degradation rate after the induction period decreased to $^1/_2$ its prior value. For the 10° decrease from 60 to 50° the similarly estimated linear degradation rate is decreased to $^1/_{10}$ its prior value. This indicates that the apparent overall heat of activation for the processes of pyrolytic degradation is of high magnitude. If a decrease to $^1/_{10}$ the value is postulated for the next 10° decrease in temperature, infrared assay should not be able to demonstrate any significant pyrolytic degradation for more than a year at 40°. Pure, dry aspirin anhydride should be indefinitely stable at 30° by this criterion. Consideration of the additional phenomenon of the lengthening induction period with lowered temperature makes this estimate a conservative one.

Proposed Mechanism for the Pyrolytic Degradation of Aspirin Anhydride.—Aspirin anhydride (I) with high moisture content (lot I) produced acetylsalicoylsalicylic acid (VII) and acetic acid (VIII) in large yield as the final products of pyrolytic degradation.



I

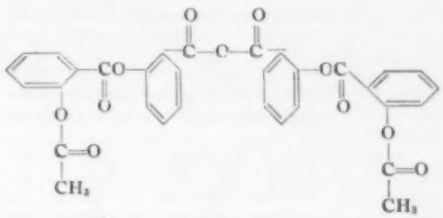


VII

VIII

The infrared spectra of the carbonyl region for aspirin anhydride pyrolytically degraded were highly consistent with the carbonyl region spectra of VII plus a partial contribution from the carbonyl spectra of acetic acid which was also present. This is clearly shown in Fig. 5.

When large amounts of dry aspirin anhydride were heated at 70° for a period of a week, a melt resulted. The carbonyl region spectra were not consistent with VII but with the anhydride of acetylsalicoylsalicylic acid (V) i. e., 2,2′-bis(2-acetoxybenzoyloxy)-benzoic anhydride.



V

Initial opinion as based on the I. R. was that the melt was slightly impure aspirin anhydride, I, since the 1,787.5 cm.$^{-1}$ band was still prominent although reduced in intensity and no band had appeared at 1,700 cm.$^{-1}$ which had been characteristic of the pyrolytic end products, i. e., VII (see Fig. 2). This is, of course, inconsistent with the fact of liquefac-

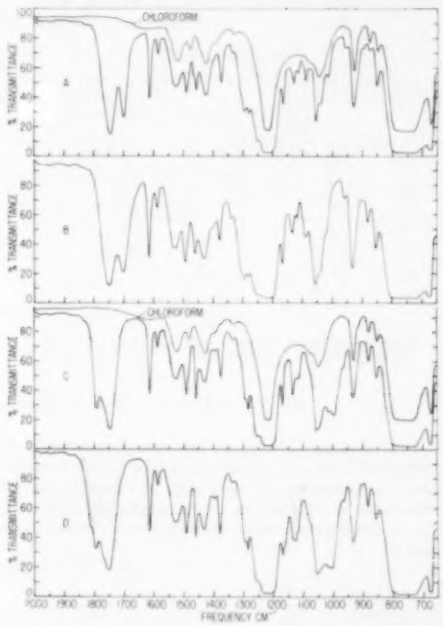


Fig. 9.—Partial infrared spectra in chloroform solution, concentration 0.5%. A.—acetylsalicoylsalicylic acid. B.—aspirin anhydride, lot I, completely degraded at 70°. C.—2,2′-Bis(2-acetoxybenzoyloxy)-benzoic anhydride. D.—anhydrous aspirin anhydride degraded at 70° for forty-eight hours in the presence of powdered soft glass. The dashed line is the absorption of the mixed anhydride of aspirin and acetic acid.

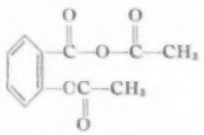
tion. However, Fig. 6 clearly shows that the carbonyl infrared of this large anhydride V is consistent with the postulate that the melt is a mixture of I and V. Comparison of the curve of Fig. 6 with those of Fig. 2 shows that V is consistent with the carbonyl spectra of partially degraded material.

The possibility of the existence of V as an intermediate in the degradation of moist aspirin anhydride is also consistent with the forms of the rate plots of degradation, particularly in Fig. 4. The spectra at 1,700 cm.$^{-1}$ continue to show large changes when that at 1,737.5 cm.$^{-1}$ (or even at 1,787.5 cm.$^{-1}$ in Fig. 3) shows small or none. This indicates the production of an intermediate, probably V, which is again transformed, probably by water to VII.

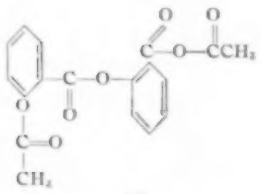
The degradation of the wetter lot I differed from lot II by showing a more definitive change in absorbance for 1,700 and 1,737.5 cm.$^{-1}$. The absorbance of lot II remained at much lower values than for lot I at these frequencies.

In a specific case where dry, pure aspirin anhydride (lot VII) was admixed with powdered soft glass and heated at 70°, liquefaction occurred readily. After a week, a chloroform solution of material showed a definite shoulder at 1,800 cm.$^{-1}$, a band of much higher frequency than in the carbonyl region of compounds I, V, and VII.

The possible derivative of aspirin anhydride with such a frequency was found to be the mixed anhydride of acetic acid and acetylsalicylic acid, II.

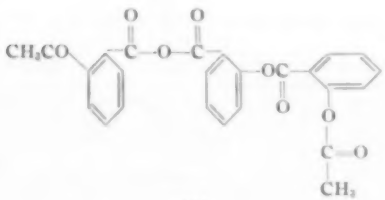


II



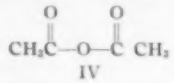
VI

It is also probable that the mixed anhydride of acetic acid and acetylsalicoylsalicylic acid VI would have a similar carbonyl spectrum and that the mixed anhydride of aspirin and acetylsalicoylsalicylic acid III would have a carbonyl spectrum similar to I and V.



III

Acetic anhydride (IV) is also a product of the pyrolytic degradation of aspirin anhydride.



IV

A mechanism for the pyrolytic degradation of aspirin anhydride consistent with the above facts would be acyl interchange with the ultimate products prior to hydrolyis, being acetic anhydride (IV) and the anhydride of acetylsalicoylsalicylic acid (V). A scheme which could represent all possible acyl exchanges is given in Fig. 10 where the solid lines represent the consequences of acyl exchange of the designated compound with aspirin anhydride, which should be in excess throughout the major part of the degradation. The two arrows from each compound represent the products of exchange. The reverse of the arrowed directions give the other possibilities, where one of the products would be aspirin anhydride and the other would be the compound at the origin of the arrows. The dashed lines represent the two products of acyl interchange of a compound with itself.

Of course, intramolecular acyl exchange is also possible with the monomolecular product of I being the mixed anhydride of acetic acid and acetylsalicoylsalicylic acid (VI).

The subsequent products of acyl exchange could be as diagrammed in Fig. 11. Two additional possible exchanges not covered by Figs. 10 and 11 are: VI + II ⟶ III + IV and VI + III ⟶ I + V.

The buildup of the anhydride of acetylsalicoylsalicylic acid (VI) and acetic anhydride (IV) as products of pyrolytic degradation can be readily concluded from these charts. The presence of extremely small amounts of water could yield acetylsalicoylsalicylic acid (VII) and acetic acid (VIII), since IV and V in the "melt" are readily hydrolyzed.

Further Evidence for Acetylsalicoylsalicylic Acid (VII) and 2,2′-Bis(2-acetoxybenzoyloxy)-benzoic Anhydride (V) as End Products of Aspirin Anhydride Pyrolysis.—The complete infrared spectra in chloroform solution of acetylsalicoylsalicylic acid (VII)

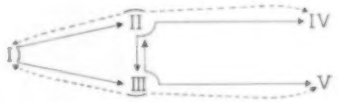


Fig. 10.—The products of acyl exchange. The solid arrows represent the two products of acyl exchange of a compound with aspirin anhydride. The dashed arrows represent the two products of acyl exchange of a compound with itself.

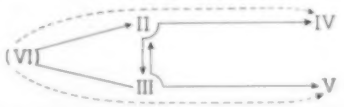


Fig. 11.—The products of acyl exchange from an initial intramolecular exchange, I → VI. The solid arrows represent the two products of acyl exchange of a compound with aspirin anhydride. The dashed arrows represent the two products of acyl exchange of a compound with itself.

and the end products from moist aspirin anhydride, lot I, degradation at 70°, are identical (see Fig. 9, A and B).

In addition, acetylsalicoylsalicylic acid (VII) was isolated from this pyrolyzed aspirin anhydride and identified.

A comparison of the spectra in chloroform solution of 2,2′-bis(2-acetoxybenzoyloxy) benzoic anhydride (V) and aspirin anhydride (I), degraded at 70° for forty-eight hours, was almost identical. The slight spectral difference in the degraded aspirin anhydride spectrum can be explained by the presence of a small amount of mixed anhydride such as the mixed anhydride of aspirin and acetic acid (II) (see Fig. 9, C and D).

The dashed line in Fig. 9D shows the spectra of the mixed anhydride of aspirin and acetic acid (II). It is readily seen that the additional high-frequency carbonyl band at 1,805 cm.$^{-1}$ in the degradation product can be attributed to intermediate mixed anhydrides. The mixed anhydrides of aspirin-acetic acids and acetylsalicoylsalicylic-acetic acids have similar carbonyl frequencies in chloroform solution of 1,805, 1,758, 1,753, and 1,805, 1,740 cm.$^{-1}$, respectively. Both anhydrides can be represented by the dashed curve in Fig. 9D. The special drying of the aspirin anhydride prior to degradation reduced the possibility of hydrolytic decomposition, thus making it possible for the mixed anhydrides to persist longer than usual in this case. The relatively short decomposition time combined with ground "soft" glass catalysis allowed the existence of mixed anhydrides to be detected before their complete disproportionation to symmetrical anhydrides. A longer period of heating or a higher temperature would have led to complete destruction of the mixed anhydrides and the formation of the usual decomposition end products.[2]

[2] This was shown by the fact that hydrolysis of the decomposition complex by boiling a 1-Gm. sample with water for three hours resulted in a mixture of salicylic acid, aspirin, and acetylsalicoylsalicylic acid by I. R. analysis. These products support the proposed mechanism of pyrolysis.

SUMMARY

1. It is shown that dry aspirin anhydride in the absence of base at 40° and possibly also at 50° is stable indefinitely. The pyrolytic decomposition of aspirin anhydride is accelerated by alkalies or by impurities that will cause aspirin anhydride to melt and come in contact with basic surfaces, such as "soft" glass.

2. It is definitely indicated that aspirin anhydride crystals are readily solvated, that solvation enhances pyrolytic degradation. Micronization and subsequent drying favor increased stabilization to heat.

3. Pyrolytic degradation above 50° does not reflect ease of degradation below 35° since aspirin anhydride pyrolysis has a very high heat of degradation, i. e., great changes in degradation rate with temperature.

4. Infrared investigations of products, isolated and in the degrading melt, show the presence of all possible products of acyl transfer. In moist systems the end products of the pyrolysis of aspirin anhydride are acetylsalicoylsalicylic acid and acetic acid. In anhydrous systems the mixed and symmetrical anhydrides of these two acids are the major products.

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The Stability of Acetylsalicylic Acid in Suspension*

By SEYMOUR M. BLAUG and JEREMII W. WESOLOWSKI

The effect of temperature and concentration on the hydrolysis rate of acetylsalicylic acid suspensions was studied. As would be expected for a zero-order reaction the more concentrated suspensions showed a longer half-life. The effect of additives on the stability of the aqueous suspensions was evaluated. Fifty per cent (w/v) crystalline sorbitol exerted the most pronounced stabilizing action. It raised the half-life of an aqueous suspension containing 6.5 Gm. of acetylsalicylic acid in 100 ml. of water from 1,748 hours to 3,396 hours at 25°.

THE IMMENSITY of the problem involved in preparing a liquid dosage form of acetylsalicylic acid can be summed up in the observation of Friedlander and Feinberg (1) " . . . Acetylsalicylic acid should never be used in aqueous solution because of its instability." Acetylsalicylic acid in aqueous media will hydrolyze almost completely in less than one week.

Edwards (2, 3) made a thorough study of the kinetics of acetylsalicylic acid hydrolysis. He found the hydrolysis rate to be pseudo first order, the net velocity being a function of six possible reactions. The pH at which the greatest stability was obtained was between pH 2–3. This

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represents the pH of a saturated acetylsalicylic acid solution.

Garrett (4) extended Edwards' study to several acyl esters of salicylic acid in an attempt to correlate the interdependence of solubility and rate of degradation of saturated solutions. If the degradation of homogenous solution is first order, the saturated solution should be zero order.

The hydrolysis of acetylsalicylic acid depends upon the amount of acetylsalicylic acid in solution, hence suspensions of acetylsalicylic acid show a low degree of hydrolysis relative to the total amount of drug in suspension. K. C. James (5) found that a 6 per cent unbuffered aqueous acetylsalicylic acid suspension, after storage for forty-seven days at room temperature contained 89 per cent of the drug.

Various techniques have been recommended for preparing a more stable acetylsalicylic acid solution. Clark (6) developed a formula containing potassium citrate that will give a "minimum of hydrolysis." Organic solvents such as ethanol, propylene glycol, and glycerin solubilize the acid, but the disadvantage to their use lies in the fact that most patients will not find any of these solvents palatable. Schwarz, *et al.* (7), have developed an acetylsalicylic acid elixir which, according to the authors, holds promise as a pediatric preparation. The formula recommended uses as solvent a blend of ethanol and polyethylene glycol 400. At the end of twenty-four days, 17.7 per cent of the acetylsalicylic acid hydrolyzed in this solvent.

A recent formulation (8) of an acetylsalicylic acid suspension has been found " . . . to be stable for one month without excessive hydrolysis." According to the authors, crystalline sorbitol, when used in combination with other ingredients, exerted a pronounced stabilizing effect on the suspension. Sorbitol has been used to stabilize vitamin C and B_{12} preparations (9), the action being attributed to two possible causes; the reduced availability of water in the sorbitol solution or complex formation between sorbitol and the drug.

The objectives of this investigation were to study the kinetics of acetylsalicylic acid suspensions and the effects of various additives on the stability of the suspension.

Higuchi, *et al.* (10, 11), have reported on the interaction of acetylsalicylic acid and salicylic acid with several of the additives used in this study.

EXPERIMENTAL

Reagents.—Recrystallized acetylsalicylic acid, salicylic acid, calcium gluconate; reagent grade phosphoric acid, dibasic sodium phosphate, glycerin, and commercially available crystalline sorbitol, polyethylene glycol 6000, polyvinylpyrrolidone, and N-methyl-2-pyrrolidone.

Procedure.—An absorption curve for acetylsalicylic acid and salicylic acid was prepared using buffer (pH 3.0) as the solvent. Maximum absorption at this pH occurred at 275 mμ for acetylsalicylic acid and at 298 mμ for salicylic acid.

Acetylsalicylic acid, 6.5-Gm. portions, 100 mesh and 60 mesh, were accurately weighed into 125-ml. glass-stoppered wide-bottom flasks. To each flask was added 100 ml. of buffer solution composed of 3 *N* phosphoric acid adjusted to pH 3.0 with 3 *N* dibasic sodium phosphate. This pH was chosen because it is the pH of a saturated acetylsalicylic acid solution. At pH 3.0 the solubility of acetylsalicylic acid is approximately 4.22 Gm./L. (4). Buffer solutions were added at the same temperatures used in the hydrolysis study.

The flasks were immersed in a mechanical shaker constant temperature bath accurate to ±0.5°. The hydrolysis rate was studied over a twenty-four-hour period at temperatures of 46, 50, and 60° by withdrawing aliquot portions of the supernatant liquid at zero time and at four-hour intervals thereafter, using a sintered-glass funnel attached to a pipet, diluting with buffer solution, and analyzing for

TABLE I.—THE EFFECT OF TEMPERATURE ON THE HYDROLYSIS RATE OF ACETYLSALICYLIC ACID SUSPENSIONS[a]

	Mesh Size	Temperature, °C.	$k \times 10^3$	$t^1/_2$ Hr.	ΔF Kcal./Mol.	ΔHa KCal.	ΔS Cal./Deg./Mole
A	60	25[b]	0.154	1169.0	5.204	18.472	44.52
	100		0.150	1200.0	5.221	18.872	44.80
B	60	46	0.867	207.6	4.474	18.472	43.88
	100		0.870	206.9	4.470	18.872	45.14
C	60	50	1.71	105.2	4.093	18.472	44.52
	100		1.76	102.2	4.075	18.872	45.81
D	60	60	7.16	25.1	3.273	18.472	45.64
	100		7.35	24.5	3.255	18.872	46.89
E	60	25[b]	0.273	1321.4	4.863	13.472	28.89
F	60	46	0.913	395.1	4.441	13.472	28.31
G	60	50	1.58	228.3	4.144	13.472	28.88
H	60	60	5.37	67.2	3.462	13.472	30.06

A–D contain 6.5 Gm. acetylsalicylic acid/100 ml. E–H contain 13.0 Gm. acetylsalicylic acid/100 ml.
[a] The ΔH ionization for water was calculated to be 12.928 Kcal. in the temperature range studied.
[b] Values at 25° calculated from knowledge of ΔHa.

salicylic acid spectrophotometrically at 298 mμ. The same procedure was repeated on suspensions containing 13.0 Gm. of 60-mesh acetylsalicylic acid. All suspensions were prepared in duplicate. Results are shown in Table I and in Figs. 1, 2, and 3. The thermodynamic values reported in Table I were obtained from plotting log k *vs.* $1/T$ and calculated from the following equations:

$$(a) \qquad \Delta F = -RT \ln K$$

$$(b) \qquad \frac{\Delta F - \Delta H}{T} = -\Delta S$$

The effect of additives on the stability of acetylsalicylic acid suspensions was evaluated by preparing varying per cent solutions of the agents under investigation in the buffer solution. Glycerin and sorbitol were also prepared in distilled water in order to explain more fully the reported (8) stabilizing action of sorbitol on acetylsalicylic acid suspensions.

Acetylsalicylic acid, 6.5-Gm. portions, 60-mesh, were accurately weighed into 125-ml. glass-stoppered wide-bottom flasks. To each flask was added 100 ml. of solution of the agent under investigation. Solutions were added at 50° and the flasks were immersed in a mechanical shaker constant temperature bath adjusted to 50° (±0.5°). All suspensions were prepared in duplicate.

Aliquot portions of the supernatant liquid were removed at zero time and at four-hour intervals thereafter and diluted with buffer solution to a volume which enabled spectrophotometric determination of salicylic acid. Samples were taken over a twenty-four-hour period. Agents studied and results are shown in Table II and in Fig. 4.

TABLE II.—EFFECT OF ADDITIVES ON THE HYDROLYSIS OF ACETYLSALICYLIC ACID SUSPENSIONS[a]

Compound	Concentration, (w/v) %	$k \times 10^{5}$[c]	$t_{1/2}$ Hr.[d]
Distilled water	..	1.21	149
Buffer pH 3.0	..	1.76	102
Calcium gluconate[b]	1	1.93	93.3
Glycerin	50	3.42	52.6
Glycerin[b]	50	1.32	136.5
N-Methyl-2-pyrrolidone	4	1.78	90
Polyethylene glycol 6000	3	0.385	468
Polyethylene glycol 6000	2	0.477	378
Polyethylene glycol 6000	1	1.0	180
Polyvinylpyrrolidone	1	0.435	414
Polyvinylpyrrolidone	0.25	0.98	204
Polyvinylpyrrolidone	0.1	1.27	142
Polyvinylpyrrolidone	1	..	..
N-Methyl-2-pyrrolidone	4	0.60	300
Polyvinylpyrrolidone	1	..	..
N-Methyl-2-pyrrolidone	2	0.57	316
Salicylic acid	0.01	1.74	103.5
Sorbitol, crystalline	50	1.36	132.5
Sorbitol, crystalline[b]	50	0.625	288

[a] 6.5% suspensions of 100-mesh acetylsalicylic acid prepared in pH 3.0 buffer at 50° except where otherwise noted.
[b] Prepared in distilled water.
[c] Obtained from a plot of acetylsalicylic acid concentration *vs.* time.
[d] $t_{1/2} = a/2k$ (a = initial acetylsalicylic acid concentration).

DISCUSSION

Acetylsalicylic acid suspensions prepared in an aqueous solvent exhibited a longer half-life than the corresponding suspension prepared in a pH 3.0 buffer. This was due to the lower final pH (2.5) of the unbuffered suspension.

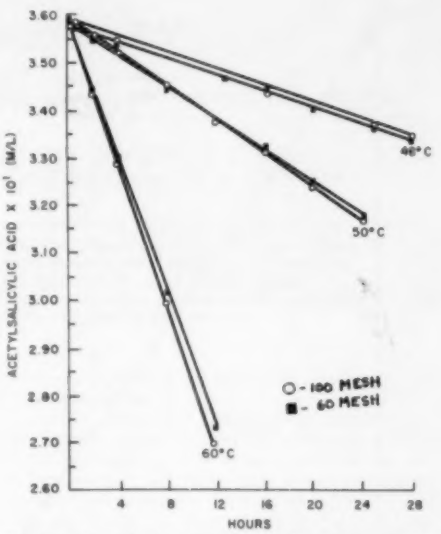

Fig. 1.—Hydrolysis of acetylsalicylic acid suspension (6.5% w/v).

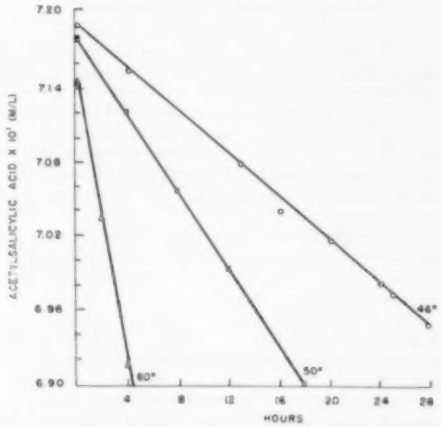

Fig. 2.—Hydrolysis of acetylsalicylic acid suspension (13% w/v).

It was found that the polyethylene glycol 6000 and polyvinylpyrrolidone exhibited apparently dramatic stabilizing effects on acetylsalicylic acid in suspension. However, on closer investigation it was found that an insoluble gummy precipitate formed which was very difficult to redisperse. Previous workers (10, 11) reported that P. E. G. 6000 and polyvinylpyrrolidone formed soluble complexes with salicylic acid at 30°. At the temperature used in this study the complex formed probably has a lower solubility than it does at 30°. This will be verified in another study now under way. Therefore, the hydrolysis constants obtained are only indicative of the salicylic acid not present in the insoluble complex.

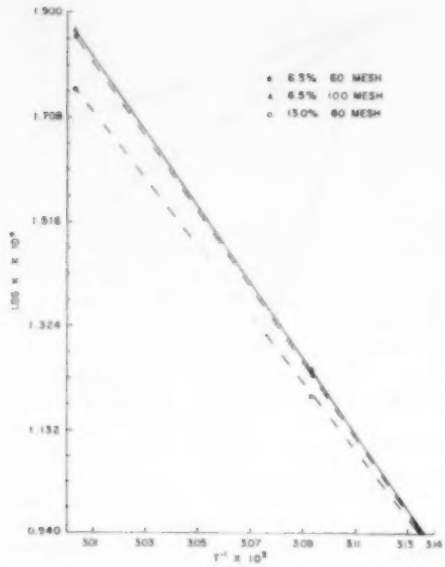

Fig. 3.—The effect of temperature on the hydrolysis rate of acetylsalicylic acid suspensions.

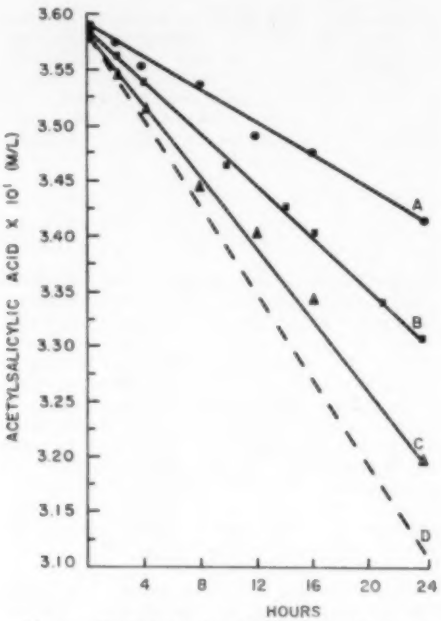

Fig. 4.—Hydrolysis of acetylsalicylic acid suspension at 50° in: A, 50% sorbitol (w/v) in water. B, water alone. C, 50% sorbitol (w/v) in pH 3.0 buffer. D, pH 3.0 buffer alone.

N-Methyl-2-pyrrolidone enhanced the solubility of acetylsalicylic acid, therefore an increase in hydrolysis rate was observed.

Calcium gluconate accelerated the hydrolysis of acetylsalicylic acid in suspension because of the high pH of the aqueous solution. This was contrary to the reported (12) stabilizing action of calcium gluconate on aqueous acetylsalicylic acid preparations.

Acetylsalicylic acid suspensions were prepared in which the supernatant liquid was saturated with salicylic acid. This procedure was without effect on the hydrolysis rate.

The effect of glycerin was studied in aqueous and buffered suspensions. In both cases glycerin was found to increase the hydrolysis rate to a significant degree. Since the hydrolysis rate is directly proportional to the concentration of acetylsalicylic acid in solution, the increased hydrolysis rate was probably due to the better solvent action of the glycerin-water solution.

Fifty per cent crystalline sorbitol in aqueous acetylsalicylic acid suspension exerted a pronounced stabilizing action. The half-life was raised from 1,748 hours to 3,396 hours at 25°. Even in the buffered suspension there was some stabilizing action but it was not as great as in the unbuffered suspension. A separate investigation is being conducted on the action of sorbitol on the stability of several medicaments.

As would be expected for a zero-order reaction the more concentrated suspension showed a longer half-life. Since the hydrolysis rate is independent of the total acetylsalicylic acid concentration and dependent on the amount of acetylsalicylic acid in solution, the acetylsalicylic acid hydrolysis would be smaller in proportion to the total acetylsalicylic acid concentration, for the more concentrated suspension.

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Steroidal Sapogenins LV*

Survey of Plants for Steroidal Sapogenins and Other Constituents

By MONROE E. WALL, C. S. FENSKE, J. W. GARVIN, J. J. WILLAMAN, QUENTIN JONES, BERNICE G. SCHUBERT, and H. S. GENTRY

This report covers the fifth 1,000 accessions in a survey of plants for steroidal sapogenins and is thus a continuation of the previous four reports (1–7). Data are given for 990 accessions (all but 107 from the United States), representing 147 families, 547 genera, and 921 species. Few steroidal sapogenins were found, largely because of the absence of *Agave*, *Yucca*, and *Dioscorea;* but alkaloids were qualitatively identified in 86 species, 45 of which are new to the record.

THIS IS A CONTINUATION of the previous reports (1–7) on the first four 1,000 accessions. It covers the fifth 1,000 plant samples in our survey of the plant world for steroidal sapogenins, and includes qualitative results for flavonoids, alkaloids, tannins, and unsaturated sterols.

PROCUREMENT

By 1954 the search for plant sources of steroidal sapogenins by the three cooperating federal agencies had been in operation for approximately four years. Leads developed during this period indicated that the three genera, *Dioscorea*, *Agave*, and *Yucca*, were the most promising sources of useful steroidal sapogenins. Procurement activities in the latter part of this period reflected the attention focused on these genera and their near relatives. Representatives of many other families were examined but the major emphasis became centered on the *Dioscoreaceae*, *Amaryllidaceae*, and *Liliaceae*.

The present report chronicles a different phase of of the program. Species of *Dioscorea* had been settled upon as having the most potential for becoming crop sources of cortical and hormonal analogs. Intensive crop development studies by the Crops Research Division, guided in the selection of superior strains by chemical evaluations supplied by the Eastern Utilization Research and Development Division, had naturally become an increasingly important phase of the program. But because *Dioscorea*, *Agave*, and *Yucca* had by this time been rather thoroughly screened for steroidal sapogenins, procurement activities were directed once more toward a broad survey of the higher plants with the hope of finding a steroid-rich species more amenable to crop conditions of the United States than are species of *Dioscorea*.

The logical place to look for species which are apt to fit crop environments of this country is in our native flora. Of the 990 accessions here reported on, all but 107 were collected in the United States. Collecting was concentrated in the Atlantic States as evidenced by over 800 accessions here listed from that area.

Another reflection of the broadness of the screening program during this period is the large number of families, genera, and species represented. Included are 921 species in 547 genera distributed through 147 families. This same philosophy of obtaining broad coverage of our native flora continues with other areas of the country receiving increasingly more emphasis in our procurement activities.

REVISED ALKALOID PROCEDURE

In previous reports we were conducting the Mayer alkaloid tests on samples in which each ml. of final test solution was equivalent to 0.2 Gm. of original dry plant sample. Because we were obtaining a much larger proportion of negative alkaloid tests than expected from statistical averages, we have greatly increased the concentrations used so that 1 ml. of final test solution is equivalent to 4.0 Gm. of dry plant material. Thus the revised procedure is much more sensitive for the purposes of alkaloid screening.

RESULTS

A compilation of all data is given in Table I, page 696, *et seq.* Since in the past steroidal sapogenins were found mostly in *Agave*, *Yucca*, and *Dioscorea*, and since in the present collection these genera were represented by only six collections, the table, as previously used, has been modified by the omission of the columns concerning sapogenins. Their presence is indicated by footnote *b* and the details are reported in the text.

Saponins, as indicated by hemolysis, were found in only 10% of the species. This is a surprisingly low incidence, since in the previous 4,000 accessions the occurrence was 35% of the species exclusive of those in *Agave*, *Dioscorea*, and *Yucca*. The following list gives the information on the steroidal sapogenins found; the accession number, the plant, the plant part, the amount, and kind of sapogenins: 4127, 4705 *Dioscorea deltoidea* (*Dioscoreaceae*); r, t; 0.5 and 1.2%; diosgenin. 4138 *Dioscorea quaternata;* r; 0.5%; diosgenin. 4653 *Clintonia umbellulata* (*Liliaceae*); l, s, r; 0.1%; not determined. 4678 *Trillium flexipes* (*Liliaceae*); l, s, fr; 0.3%; not identified. 4713B *Cestrum* sp. (*Solanaceae*); fr; 0.5%; tigogenin. 4743 *Tribulus terrestris* (*Zygophyllaceae*); l, s, fl; 0.3%; tigogenin.

* Received August 4, 1959, from M. E. Wall, C. S. Fenske, J. W. Garvin, and J. J. Willaman of Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Dept. of Agriculture, Philadelphia 18, Pa., and Quentin Jones, Bernice G. Schubert, and H. S. Gentry of New Crops Research Branch, Crops Research Division, Agricultural Research Service, U. S. Dept. of Agriculture, Beltsville, Md.

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TABLE I.—CONSTITUENTS FOUND IN THE PLANT COLLECTIONS

Code for Source Names

C. G. F.—U. S. Plant Introduction Garden, Coconut Grove, Fla. (cult.)
Chico, Calif.—U. S. Plant Introduction Garden, Chico, Calif. (cult.)

Code for Plant Parts

b—bark
bu—bulb
fl—influorescence
fr—fruit
l—leaf
r—root
s—stem
t—tuber
tw—twig
w—whole plant above ground

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			ACANTHACEAE						
4028	*Aphelandra nitens*	Tingo Maria, Peru	4/54	l, s, r	+[a]	0	0	0	+++
4253	*Dianthera (Justicia) Americana*	Johnston Co., N. C.	6/54	l, s	−	0	0	0	0
			ACERACEAE						
4252	*Acer Floridanum*	Wake Co., N. C.	6/54	l, s	−	0	0	+	0
4510	*Acer Pensylvanicum*	Haywood Co., N. C.	7/54	l, s, fr	−	+	0	+++	..
			AIZOACEAE						
4588	*Mollugo verticillata*	Brunswick, N. C.	7/54	l, s, r	+[a]	0	0	0	..
4680	*Mollugo verticillata*	Dorchester Co., Md.	7/54	l, s, fl, r	−	0	0	++	..
4897	*Sesuvium portulacastrum*	Franklin Co., Fla.	8/54	l, s	−	0	0	0	..
			ALISMACEAE						
4689	*Alisma subcordatum*	Dorchester Co., Md.	7/54	l, s, fl, r	−	0	0	0	..
4439	*Lophotocarpus calycinus*	Brunswick Co., N. C.	7/54	l, s, fr, r	−	0	0	0	..
4847	*Sagittaria engelmanniana*	Wayne Co., Pa.	8/54	l, s, fl, r	−	0	0	0	..
4744	*Sagittaria falcata*	Carteret Co., N. C.	7/54	l, s	−	0	0	0	..
4668	*Sagittaria latifolia*	Baltimore Co., Md.	7/54	l, s, r	−	0	0	0	..
			AMARANTHACEAE						
4747	*Acnida cannabina*	Carteret Co., N. C.	7/54	l, s	+[a]	0	0	0	..
4688	*Acnida cannabina*	Dorchester Co., Md.	7/54	l, s, r	−	0	0	0	..
4197	*Alternanthera philoxeroides*	New Hanover Co., N. C.	6/54	l, s, fl, r	+[a]	0	0	0	++
4682	*Amaranthus graecizans*	Dorchester Co., Md.	7/54	l, s, fl, r	−	0	0	0	..
4478	*Amaranthus hybridus*	Nansemond Co., Va.	7/54	l, s, r	+[a]	0	0	0	..
4732	*Amaranthus spinosus*	New Hanover Co., N. C.	7/54	l, s, fl, r	+[a]	0	0	0	..
4892	*Froelichia Floridana*	Leon Co., Fla.	8/54	l, s, fl, r	−	0	0	0	..
			ANACARDIACEAE						
4153	*Pistacia lentiscus*	Chico, Calif.	5/54	l, s	−	+++	0	+++	+
4051	*Rhus aromatica*	Hancock Co., Ga.	4/54	l	−	0	0	+	+
4073	*Rhus copallina*	McIntosh Co., Ga.	4/54	l	−	0	0	+++	++
4082	*Rhus glabra*	Clarke Co., Ga.	4/54	l	−	0	0	++	0
4525	*Rhus glabra*	Buncombe Co., N. C.	7/54	s, fr	−	0	0	+++	..
4787	*Rhus ovata*	Aguanga, Calif.	7/54	l	−	0	0	+++	..
4659	*Rhus typhina*	Garrett Co., Md.	7/54	l, s, fl	−	0	0	+++	..
4286	*Rhus vernix*	Anne Arundel Co., Md.	6/54		−	0	0	0	..

			ANNONACEAE					
4386	*Annona cherimola*	C. G. F.	7/54	l, s	–	0	++	0	..
4716	*Annona glabra*	C. G. F.	7/54	l, s, fr	–	0	++	0	..
4717	*Annona reticulata*	C. G. F.	7/54	l, s	–	0	++	0	..
4347	*Asimina pygmaea*	Leon Co., Fla.	6/54	l, s, fr	–	0	0	0	..
4221	*Asimina triloba*	Wake Co., N. C.	6/54	l, s	–	0	0	0	0
			APOCYNACEAE					
4377	*Adenium obesum*	C. G. F.	7/54	l, s	–	0	0	0	..
4712	*Alamanda violacea*	C. G. F.	7/54	l, s	–	0	0	0	..
4052	*Amsonia ciliata*	Johnson Co., Ga.	4/54	l	–	0	+	0	++
4828	*Apocynum androsaemifolium*	Cayuta Lake, N. Y.	8/54	l, s, fl, r	–	0	0	0	..
4065	*Apocynum cannabinum*	McIntosh Co., Ga.	4/54	l	–	0	0	0	++
			AQUIFOLIACEAE					
4453	*Ilex cassine*	Brunswick Co., N. C.	7/54	l, s, fr	–	0	+	0	..
4265	*Ilex vomitoria*	Brunswick Co., N. C.	6/54	l, s, r	–	0	0	0	..
			ARACEAE					
4216	*Arisaema triphyllum*	Wake Co., N. C.	6/54	l, s, fr, bu	–	0	0	0	0
4041	*Colocasia sp.*	Tingo Maria, Peru	4/54	l, s	–	0	0	0	+
4402	*Orontium aquaticum*	Atlantic Co., N. J.	7/54	l, s, fl, r	–	0	0	0	..
4745	*Peltandra Virginica*	Carteret Co., N. C.	7/54	l, s, fr	–	0	0	0	..
4151	*Symplocarpus foetidus*	Grant Co., W. Va.	5/54	l, s, r	+*	0	0	0	+
			ARALIACEAE					
4656	*Aralia nudicaulis*	Monongalia Co., W. Va.	7/54	l, s, fr	–	0	0	0	..
4561	*Aralia racemosa*	Macon Co., N. C.	7/54	l, s, r	–	0	0	0	..
4605	*Aralia racemosa*	Clay Co., W. Va.	7/54	l, s, fl, r	–	0	0	0	..
4304	*Aralia spinosa*	Lancaster Co., Va.	6/54	l, s	–	0	0	0	..
			ARISTOLOCHIACEAE					
4596	*Aristolochia durior*	Haywood Co., N. C.	7/54	l, s	–	0	0	0	..
4604	*Aristolochia durior*	Clay Co., W. Va.	7/54	l, s, fr	–	0	0	0	..
4148	*Asarum canadense*	Preston Co., W. Va.	5/54	l, s, r	–	0	+	0	+
4220	*Asarum reflexum*	Wake Co., N. C.	6/54	l, s, r	–	0	0	0	0
			ASCLEPIADACEAE					
4628	*Ampelamus albidus*	Union Co., Ky.	7/54	l, s, fl, r	–	0	0	0	..
4334	*Asclepias amplexicaulis*	Wake Co., N. C.	6/54	l, s, fr, r	+*	0	0	0	..
4799	*Asclepias Californica*	Dripping Springs, Calif.	7/54	w	+*	0	0	0	..
4079	*Asclepias humistrata*	Bryan Co., Ga.	4/54	l	–	0	0	0	++
4685	*Asclepias incarnata*	Dorchester Co., Md.	7/54	l, s, fl, r	–	0	0	0	..
4904	*Asclepias cf. obovata*	Franklin Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
4490	*Asclepias syriaca*	Nansemond Co., Va.	7/54	l, s, fr	–	0	0	0	..
4245	*Asclepias tuberosa*	Johnston Co., N. C.	6/54	l, s, fl, r	–	0	0	0	+++
4928	*Asclepias verticillata*	Charles Co., Md.	8/54	l, s, r	–	0	0	0	..
4777	*Asclepias (Acerates) viridiflora*	Baltimore Co., Fla.	7/54	l, s, fr, r	–	+	0	0	..
4750	*Gonolobus suberosus*	Carteret Co., N. C.	7/54	l, s, fr	–	0	0	0	..
			BALSAMINACEAE					
4247	*Impatiens cadensis (I. biflora)*	Wake Co., N. C.	6/54	l, s	+*	0	0	0	+

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
		BEGONIACEAE							
4008	*Begonia foliosa* var. *Australis*	Cayumba, Tingo Maria, Peru	4/54	l, s, r	−	0	0	0	0
		BERBERIDACEAE							
4530	*Akebia quinata*	Buncombe Co., N. C.	7/54	l, s	−	0	0	0	..
4546	*Berberis thunbergii*	Henderson Co., N. C.	7/54	l, s	−	0	+++	0	..
4529	*Caulophyllum thalictroides*	Buncombe Co., N. C.	7/54	l, s, fr, r	+[a]	0	++	0	..
4551	*Diphylleia cymosa*	Macon Co., N. C.	7/54	l, s, fr	−	+++	0	0	..
		BETULACEAE							
4507	*Betula lenta*	Haywood Co., N. C.	7/54	l, s	−	0	0	+	..
4083	*Betula nigra*	Clarke Co., Ga.	4/54	l	+[a]	0	0	0	0
4845	*Betula populifolia*	Wayne Co., Pa.	8/54	l, s	−	0	0	++	..
4325	*Carpinus Caroliniana*	Chatham Co., N. C.	6/54	l, s	−	0	0	0	..
4393	*Corylus Americana*	Baltimore Co., Md.	7/54	l, s	−	0	0	0	..
4526	*Ostrya Virginiana*	Buncombe Co., N. C.	7/54	l, s	−	+	0	0	..
4666	*Ostrya Virginiana*	Allegany Co., Md.	7/54	l, s	−	0	0	0	..
		BIGNONIACEAE							
4719	*Arrabidaea rotundata*	C. G. F.	7/54	l, s	−	0	0	0	..
4611	*Bignonia capreolata*	Floyd Co., Ky.	7/54	l, s	+[a]	0	0	0	..
4365	*Catalpa speciosa*	Jefferson Co., Ga.	6/54	l, s	−	0	0	0	..
4089	*Campsis radicans*	Clarke Co., Ga.	4/54	l, s	−	0	0	0	+++
4923	*Campsis radicans*	Charles Co., Md.	8/54	fr	−	0	0	0	..
		BIXACEAE							
4009	*Bixa orellana*	Cayumba, Tingo Maria, Peru	4/54	l, s	−	0	0	+++	++
		BORAGINACEAE							
4025	*Cordia sp.*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	0
4806	*Cynoglossum officinale*	Yates Co., N. Y.	8/54	l, s, fl, r	−	0	0	0	..
4102	*Cynoglossum Virginianum*	Wake Co., N. C.	4/54	l, r	−	0	0	0	0
4204	*Echium vulgare*	Wake Co., N. C.	6/54	l, s, fl	−	0	0	0	0
4501	*Heliotropium anchusaefolium*	Marlboro Co., S. C.	7/54	l, s, fl	−	0	+	0	..
4638	*Heliotropium tenellum*	Bullitt Co., Ky.	7/54	l	+[a]	0	0	0	..
		BROMELIACEAE							
4756	*Tillandsia usneoides*	Carteret Co., N. C.	7/54	l, fl	−	+	0	0	..
		BUXACEAE							
4615	*Pachysandra procumbens*	Laurel Co., Ky.	7/54	l, s, r	−	0	0	0	..
		CACTACEAE							
4295	*Opuntia humifusa*	Anne Arundel Co., Md.	6/54	w	−	0	0	0	..
		CAMPANULACEAE							
4652	*Campanula Americana*	Gilmer Co., W. Va.	7/54	l, s, fl, r	−	0	0	0	..
4808	*Campanula rapunculoides*	Yates Co., N. Y.	7/54	l, s, fl, r	−	0	0	0	..
4292	*Specularia perfoliata*	Charles Co., Md.	6/54	l, s, fl, r	−	0	0	0	..
4056	*Specularia perfoliata*	McIntosh Co., Ga.	4/54	l	−	0	0	0	..

			CANNACEAE						
4763	*Canna flaccida*	Carteret Co., N. C.	7/54	l, s, fr, r	−	0	0	0	..
			CAPPARIDACEAE						
4786	*Cleome spinosa*	Baltimore Co., Md.	7/54	l, s, r	+[a]	0	0	0	..
4835	*Polanisia graveolens*	Mecklenberg, N. Y.	8/54	l, s, fr, r	−	+	+	0	..
			CAPRIFOLIACEAE						
4881	*Abelia grandiflora* (cult.)	Leon Co., Fla.	8/54	l, s, fl	−	0	0	0	..
4810	*Diervilla lonicera*	Yates Co., N. Y.	8/54	l, s, fr, r	−	0	0	0	..
4552	*Diervilla sessilifolia*	Macon Co., N. C.	7/54	l, s, fl	−	0	0	0	..
4249	*Lonicera sempervirens*	Wake Co., N. C.	6/54	l, s	−	0	0	0	..
4640	*Symphoricarpus orbiculatus*	Bullitt Co., Ky.	7/54	l, s, fl, r	−	0	0	0	..
4853	*Viburnum acerifolium*	Federick Co., Md.	8/54	l, s, r	−	0	0	++	..
4842	*Viburnum cassinoides*	Wayne Co., Pa	8/54	l, s, fr	−	0	0	0	..
4831	*Viburnum dentatum*	Cayuta Lake, N. Y.	8/54	l, s	−	0	0	+	..
4830	*Viburnum opulus*	Yates Co., N. Y.	8/54	l, s, fr	−	0	0	0	..
4092	*Viburnum rufidulum*	Clarke Co., Ga.	4/54	l	−	0	0	0	++
4829	*Viburnum sp.*	Cayuta Lake, N. Y.	8/54	l, s	−	0	0	0	..
			CARYOPHLLACEAE						
4078	*Arenaria Caroliniana*	Bryan Co., Ga.	4/54	l	+[a]	0	0	0	0
4693	*Dianthus armeria*	Accomac, Va.	7/54	l, s, fl, r	+[a]	0	0	0	..
4895	*Odontonychia erecta*	Franklin Co., Fla.	8/54	l, s, fl, r	−	0	0	0	..
4608	*Paronychia Canadensis*	Wyoming Co., W. Va.	7/54	l, s, r	−	0	0	++	..
4883	*Paronychia fastigiata*	Leon Co., Fla.	8/54	l, s, fl	−	0	0	0	..
4983	*Paronychia herniarioides*	Liberty Co., Fla.	8/54	l, s, fl	−	0	0	++	..
4090	*Saponaria officinalis*	Clarke Co., Ga.	4/54	l	+[a]	0	0	0	+
4303	*Scleranthus annuus*	Anne Arundel Co., Md.	6/54	l, s	−	0	0	0	..
4128	*Silene Caroliniana* var. *Pensylvanica*	Washington Co., Md.	5/54	l, s	+[a]	0	0	0	0
4595	*Silene cucubalus*	Carroll Co., Md.	7/54	l, s, fl	+[a]	0	0	0	..
4517	*Silene nectiflora*	Haywood Co., N. C.	7/54	l, s, fl	−	0	0	0	..
4691	*Spergularia rubra*	Dorchester Co., Md.	7/54	l, s	+[a]	0	0	0	..
			CELASTRACEAE						
4617	*Euonymus Americanus*	Pulaski Co., Ky.	7/54	l, s, fl	−	0	0	0	..
			CHENOPODIACEAE						
4900	*Atriplex arenaria*	Franklin Co., Fla.	8/54	l, s, fl, r	−	0	0	0	..
4791	*Atriplex canescens*	Aguanga, Calif.	7/54	l	−	0	+	0	..
4690	*Atriplex patula* var. ***hastata***	Dorchester Co., Md.	7/54	l, s, r	+[a]	0	0	0	..
4748	*Atriplex patula* var. ***hastata***	Carteret Co., N. C.	7/54	l, s, fl, r	−	0	0	0	..
4789	*Chenopodium album*	Aguanga, Calif.	7/54	l	+[a]	0	0	0	..
4070	*Chenopodium ambrosioides*	McIntosh Co., Ga.	4/54	l	−	0	0	0	0
4498	*Cycloloma atriplicifolia*	Lancaster Co., S. C.	7/54	l, s, r	+[a]	0	0	0	..
4771	*Suaeda linearis*	Brunswick Co., N. C.	7/54	l, s, fl, r	−	0	+	0	..
			CISTACEAE						
4280	*Hudsonia ericoides*	Pine Barrens, N. J.	6/54	l	−	0	0	+	..
4935	*Hudsonia tomentosa*	Sussex Co., Del.	8/54	l, r	−	0	0	+	..
4911	*Lechea minor*	Hammonton, N. J.	8/54	l, s, fl, r	−	0	0	+++	..
4902	*Lechea patula*	Franklin Co., Fla.	8/54	l, s, fl, r	−	0	0	+++	..
4180	*Lechea villosa*	Southport, Brunswick Co., N. C.	6/54	l, s, r	−	0	0	0	+

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			CLETHRACEAE						
4504	*Clethra acuminata*	Buncombe Co., N. C.	7/54	l, s, fl	−	0	0	++	..
4193	*Clethra alnifolia*	Sampson Co., N. C.	6/54	l, s, fl	−	0	0	0	++
4992	*Clethra tomentosa*	Liberty Co., Fla.	8/54	l, s	−	0	0	+++	..
			COMMELINACEAE						
4759	*Commelina diffusa*	Carteret Co., N. C.	7/54	l, s, fl	−	0	0	0	..
4950	*Commelina Virginica*	Leon Co., Fla.	8/54	l, s, r	−	0	0	0	..
4057	*Tradescantia (canaliculata) ohiensis*	McIntosh Co., Ga.	4/54	l	−	0	0	0	++
			COMPOSITAE						
4593	*Acanthospermum australe*	Brunswick Co., N. C.	7/54	l, s	−	0	+	0	..
4043	*Achillea millefolium*	Wake Co., N. C.	4/54	l, s	−	0	0	0	0
4543	*Actinomeris alternifolia*	Henderson Co., N. C.	7/54	l, s	+[a]	0	+	0	..
4317	*Ambrosia artemisiifolia* var. *paniculata*	Chatham Co., N. C.	6/54	l, s	−	0	0	0	..
4277	*Ambrosia trifida*	Baltimore Co., Md.	6/54	l, s	+[a]	0	0	0	..
4281	*Ambrosia trifida*	Durham Co., N. C.	6/54	l, s, fl	+[a]	0	0	0	..
4839	*Anaphalis margaritacea*	Scranton, Pa.	8/54	l, s, fl, r	−	0	0	0	..
4130	*Antennaria plantaginifolia*	Washington Co., Md.	5/54	w	−	0	0	0	0
4301	*Anthemis arvensis*	Anne Arundel Co., Md.	6/54	l, s, fl, r	−	0	0	0	..
4279	*Arctium lappa*	Baltimore Co., Md.	6/54	l, s	+[a]	0	0	0	..
4539	*Arctium minus*	Henderson Co., N. C.	7/54	l, s, fl	−	0	+	0	..
4629	*Artemisia absinthium*	Union Co., Ky.	7/54	l, s	−	0	0	0	..
4527	*Artemisia ludoviciana*	Buncombe Co., N. C.	7/54	l, s, r	−	0	+	0	..
4793	*Artemisia tridentata* var. *angustifolia*	Dripping Spring, Calif.	7/54	l	−	0	++	0	..
4540	*Artemisia vulgaris*	Henderson Co., N. C.	7/54	l, s, r	−	0	0	0	..
4002	*Aspilia sp.*	Tingo Maria, Peru	4/54	l, s	+[a]	+	0	0	+
4307	*Aster lateriflorus*	Chatham Co., N. C.	6/54	l, s, r	−	0	0	0	..
4480	*Aster pilosus* var. *demotus*	Elizabeth City Co., Va.	7/54	l, s, fl, r	+[a]	0	0	0	..
4535	*Aster puniceus*	Buncombe Co., N. C.	7/54	l, s	−	0	0	0	..
4364	*Aster reticulatus*	Irwin Co., Ga.	6/54	l, s, fl	−	0	0	0	..
4974	*Balduina uniflora*	Wakulla Co., Fla.	8/54	l, s, fl, r	−	+++	0	0	..
4962	*Berlandiera pumila*	Leon Co., Fla.	8/54	l, s, r	−	0	0	0	..
4486	*Bidens bipinnata*	Nansemond Co., Va.	7/54	l, s, r	−	0	0	0	..
4013	*Bidens monticola*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	+++
4036	*Bidens pilosa* var. *radiata*	Tingo Maria, Peru	4/54	l, s, r	−	0	0	0	+
4873	*Bidens pilosa*	Leon Co., Fla	8/54	l, s, r	−	0	+	0	..
4990	*Bigelowia nudata*	Liberty Co., Fla.	8/54	l, s, fl, r	−	+++	+	0	..
4340	*Boltonia diffusa*	Thomas Co., Ga.	6/54	l, s, r	+[a]	0	0	0	..
4263	*Borrichia frutescens*	Brunswick Co., N. C.	6/54	l, s, fl	+[a]	0	0	0	..
4244	*Cacalia atriplicifolia*	Johnston Co., N. C.	6/54	l, s	−	0	0	0	0
4981	*Cacalia elliottii*	Liberty Co., Fla.	8/54	l, s, fl, r	−	0	0	0	..
4558	*Cacalia suaveolens*	Transylvania Co., N. C.	7/54	l, s	−	0	0	0	..
4528	*Carduus acanthoides*	Buncombe Co., N. C.	7/54	l, s, fl	−	0	+	0	..
4422	*Carphephorus tomentosus*	Brunswick Co., N. C.	7/54	l, s, fl, r	−	0	0	0	..
4520	*Centaurea maculosa*	Haywood Co., N. C.	7/54	l, s, fl, r	−	0	0	0	..
4597	*Centaurea maculosa*	Allegany Co., Md.	7/54	l, s, fl, r	−	0	+	0	..

4643	*Centaurea solstitialis*	Pendleton Co., Ky.	7/54	l, s, fl	–	0	0	0	..
4827	*Centaurea vochinensis*	Yates Co., N. Y.	8/54	l, s, fl, r	–	+++	0	0	..
4419	*Chrysopsis gossypina*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4420	*Chrysopsis graminifolia*	Brunswick Co., N. C.	7/54	l, s, fl, r	+°	0	0	0	..
5000	*Chrysopsis microcephala*	Liberty Co., Fla.	8/54	l, s, r	–	0	0	0	..
4374	*Chrysopsis pinifolia*	Moore Co., N. C.	6/54	l, s, r	+°	0	0	0	..
4570	*Chrysopsis trichophylla*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4538	*Cirsium altissimum*	Henderson Co., N. C.	7/54	l, s	–	0	0	0	..
4809	*Cirsium arvense*	Yates Co., N. Y.	8/54	l, s, fl	–	0	+	0	..
4376	*Cirsium repandum*	Scotland Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
4483	*Cirsium vulgare*	Elizabeth City Co., Va.	7/54	l, s, fl	–	0	0	0	..
4039	*Clibadium psilogynum*	Tingo Maria, Peru	4/54	l, s	–	0	0	0	+++
4982	*Coreopsis helianthoides*	Liberty Co., Fla.	8/54	l, s, r	–	0	0	0	..
4076	*Coreopsis lanceolate*	Bryan Co., Ga.	4/54	l	–	0	0	0	0
4323	*Coreopsis major* var. *stellata*	Orange Co., N. C.	6/54	l, s, fl	–	0	0	0	..
4648	*Coreopsis tripteris*	Rowan Co., Ky.	7/54	l, s, r	–	0	0	0	..
4250	*Coreopsis verticillata*	Wake Co., N. C.	6/54	l, s	–	0	0	0	0
4636	*Echinacea pallida*	Bullitt Co., Ky.	7/54	l, s, fl	+°	0	0	0	..
4647	*Echinacea purpurea*	Rowan Co., Ky.	7/54	l, s, fl, r	–	0	0	0	..
4702	*Eclipta alba*	Somerset Co., Md.	7/54	l, s, r	–	0	0	0	..
4425	*Elephantopus Carolinianus*	Brunswick Co., N. C.	7/54	l, s, r	–	0	+	0	..
4876	*Elephantopus elatus*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
4468	*Erechtites hieracifolia*	Brunswick Co., N. C.	7/54	l, s, r	–	0	+	0	..
4243	*Erigeron Canadensis*	Johnston Co., N. C.	6/54	l, s, r	–	0	0	0	0
4766	*Erigeron pusillus*	Carteret Co., N. C.	7/54	l, s, r	–	0	0	0	..
4059	*Erigeron quercifolius*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+
4960	*Eupatorium album*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
4266	*Eupatorium capillifolium*	Brunswick Co., N. C.	6/54	l, s, r	–	0	0	0	..
4361	*Eupatorium compositifolium*	Emanuel Co., Ga.	6/54	l, s, r	–	0	0	0	..
4332	*Eupatorium cordigerum*	Wake Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
4770	*Eupatorium cuneifolium*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	+++	0	0	..
4214	*Eupatorium fistulosum*	Wake Co., N. C.	6/54	l, s	–	0	0	0	0
4697	*Eupatorium hyssopifolium* (typical)	Somerset Co., Md.	7/54	l, s, fl, r	–	0	0	0	..
4698	*Eupatorium hyssopifolium* var. *laciniatum*	Somerset Co., Md.	7/54	l, s, fl, r	–	0	0	0	..
4554	*Eupatorium maculatum*	Macon Co., N. C.	7/54	l, s, r	–	0	0	0	..
4968	*Eupatorium mikanioides*	Wakulla Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
4437	*Eupatorium mohrii*	Brunswick Co., N. C.	7/54	l, s, r	–	+	+	0	..
4752	*Eupatorium pilosum*	Carteret Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4937	*Eupatorium resinosum*	Sussex Co., Del.	8/54	l, s, fl, r	–	0	+	+	..
4258	*Eupatorium rotundifolium*	Johnston Co., N. C.	6/54	l, s	–	0	++	0	..
4503	*Eupatorium rugosum* var. *roanense*	Haywood Co., N. C.	7/54	l, s, r	–	0	0	0	..
4877	*Eupatorium semiserratum*	Leon Co., Fla.	8/54	l, s, fl, r	–	+++	0	0	..
4246	*Eupatorium serotinum*	Wake Co., N. C.	6/54	l, s	–	0	0	0	0
4794	*Franseria acanthicarpa*	Dripping Spring, Calif.	8/54	l	+°	0	0	0	..
4966	*Gaillardia lanceolata*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
4282	*Galinsoga parviflora*	Baltimore Co., Md.	6/54	l, s, fl, r	+°	0	0	0	..
4450	*Gnaphalium obtusifolium*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4575	*Gnaphalium sp.*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4803	*Gutierrezia Californica*	Murrieta, Calif.	8/54	l, s	–	0	0	0	..

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
		COMPOSITAE							
4775	*Haplopappus divaricatus*	Brunswick Co., N. C.	7/54	l, s, r	+[a]	0	0	0	..
4495	*Helenium godfreyi*	Craven Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4329	*Helenium tenuifolium*	Wake Co., N. C.	6/54	l, s, fl, r	–	+	+	0	..
4331	*Helianthus microcephalus*	Orange Co., N. C.	6/54	l, s, r	–	0	0	0	..
4632	*Helianthus mollis*	Grayson Co., Ky.	7/54	l, s, r	–	0	0	0	..
4320	*Helianthus strumosus*	Chatham Co., N. C.	6/54	l, s, r	–	0	0	0	..
4471	*Helianthus tomentosus*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4545	*Helianthus tuberosus*	Henderson Co., N. C.	7/54	l, s, r	–	0	0	0	..
4819	*Helianthus sp.*	Yates Co., N. Y.	8/54	l, s, fl, r	–	0	0	0	..
4518	*Heliopsis helianthoides*	Haywood Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4565	*Heterotheca latifolia*	Spartanburg Co., S. C.	7/54	l, s, r	–	0	0	0	..
4131	*Hieracium venosum*	Washington Co., Md.	5/54	l, s	–	0	0	0	0
4392	*Hypochaeris radicata*	Hanover Co., Va.	7/54	l, s, fl, r	–	0	0	0	..
4665	*Inula helenium*	Allegany Co., Md.	7/54	l, s	–	0	0	0	..
4971	*Iva asperifolia*	Wakulla Co., Fla.	8/54	l, s, r	–	0	0	0	..
4472	*Iva imbricata*	Brunswick Co., N. C.	7/54	l, s	–	0	0	0	..
4234	*Lactuca Canadensis*	Durham Co., N. C.	6/54	l, s, fl, r	–	0	0	0	0
4278	*Lactuca scariola*	Baltimore Co., Md.	6/54	l, s	+[a]	0	0	0	..
4027	*Liabum pallatangense*	Tingo Maria, Peru	4/54	l, s	–	0	0	0	..
4891	*Liatris chapmanii*	Leon Co., Fla.	8/54	l, s, r	–	0	0	0	..
4421	*Liatris graminifolia*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	+
4890	*Liatris laevigata*	Leon Co., Fla.	8/54	l, s, r	–	0	+	0	..
4737	*Liatris secunda*	New Hanover Co., N. C.	7/54	l, s, r	–	0	0	0	..
4627	*Liatris squarrosa*	Hopkins Co., Ky.	7/54	l, s, fl, r	–	0	0	0	..
4650	*Liatris spicata*	Lawrence Co., Ohio	7/54	l, s, fl, r	–	0	0	0	..
4967	*Melanthera hastata*	Wakulla Co., Fla.	8/54	l, s, fl	–	0	0	0	..
4590	*Mikania scandens*	Brunswick Co., N. C.	7/54	l, s	–	+	0	0	..
4875	*Parthenium hysterophorus*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	+	0	..
4170	*Parthenium integrifolium*	Wake Co., N. C.	6/54	l, s, fl, r	–	0	0	0	0
4948	*Pluchea camphorata*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
4936	*Pluchea foetida*	Sussex Co., Del.	8/54	l, s, fl, r	–	0	0	+++	..
4949	*Pluchea foetida*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	+	..
4970	*Pluchea longifolia*	Wakulla Co., Fla.	8/54	l, s, fl, r	–	0	0	++	..
4757	*Pluchea purpurascens*	Carteret Co., N. C.	7/54	l, s	–	0	0	0	..
4344	*Pluchea rosea*	Leon Co., Fla.	6/54	l, s, fl, r	–	0	0	0	..
4790	*Pluchea sericea*	Aguanga, Calif.	7/54	l	–	0	0	0	..
4218	*Polymnia uvedalia*	Wake Co., N. C.	6/54	l, s, r	–	0	++	0	0
4178	*Pterocaulon undulatum*	Brunswick Co., N. C.	6/54	l, s	–	0	0	0	+
4616	*Ratibida pinnata*	Pulaski Co., Ky.	7/54	l, s, fl, r	–	0	0	0	..
4516	*Rudbeckia laciniata*	Haywood Co., N. C.	7/54	l, s, r	–	0	0	0	..
4189	*Rudbeckia serotina*	Brunswick Co., N. C.	6/54	l, s, fl, r	–	0	0	0	0
4991	*Senecio aureus*	Liberty Co., Fla.	8/54	l, s, r	–	0	++	0	..
4074	*Senecio Tomentosus*	Liberty Co., Ga.	4/54	l	–	0	0	0	++
4215	*Sericocarpus asterioides*	Wake Co., N. C.	6/54	l, s, fl, r	+[a]	0	0	0	0
4418	*Sericocarpus tortifolius*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	+	0	0	..
4321	*Silphium asteriscus*	Orange Co., N. C.	6/54	l, s, fl	+[a]	0	0	0	..

4257	*Silphium compositum*	Wake Co., N. C.	6/54	l, s	–	0	0	0	..
4637	*Silphium terebinthinaceum*	Bullitt Co., Ky.	7/54	l, s, fl	–	0	0	0	..
4291	*Silphium trifoliatum*	Quantico, Va.	6/54	l, s, fl	+[a]	0	0	0	..
4322	*Solidago altissima*	Chatham Co., N. C.	6/54	l, s, fl	+[a]	0	0	0	..
4764	*Solidago boottii* var. *Caroliniana*	Carteret Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4464	*Solidato fistulosa*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4553	*Solidago gigantea*	Macon Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4185	*Solidago graminifolia*	Brunswick Co., N. C.	6/54	l, s, r	–	0	0	0	0
4417	*Solidago minor*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4577	*Solidago odora*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4261	*Solidago pinetorum*	Johnston Co., N. C.	6/54	l, s, r	+[a]	++	0	0	..
4646	*Solidago rigida*	Rowan Co., Ky.	7/54	l, s, r	–	0	0	0	..
4484	*Solidago sempervirens* var. *Mexicana*	Elizabeth City Co., Va.	7/54	l, s, r	–	0	0	0	..
4969	*Solidago stricta*	Wakulla Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
4946	*Solidago tortifolia*	Leon Co., Fla.	8/54	l, s, r	–	0	0	0	..
4825	*Sonchus oleraceus*	Yates Co., N. Y.	8/54	l, s, fl, r	–	0	0	0	..
4029	*Spilanthes Americana*	Tingo Maria, Peru	4/54	l, s	–	0	0	0	+
4816	*Tanacetum vulgare*	Yates Co., N. Y.	8/54	l, s, fl, r	–	0	0	0	..
4796	*Tetradymia comosa*	Dripping Springs, Calif.	7/54	l	–	0	0	0	..
4692	*Tragopogon pratensis*	Accomac, Va.	7/54	l, s, fl, r	–	0	0	0	..
4496	*Trilisa odoratissima*	Pender Co., N. C.	7/54	l, s, r	–	0	0	0	..
4436	*Trilisa paniculata*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4345	*Verbesina aristata*	Leon Co., Fla.	6/54	l, s, fl, r	+[a]	0	0	0	..
4094	*Verbesina occidentalis*	Clark Co., Ga.	4/54	l	–	0	0	0	++
4375	*Vernonia angustifolia*	Scotland Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
4492	*Xanthium sp.*	Bertie Co., N. C.	7/54	l, s, r	–	0	0	0	..
		CONVOLVULACEAE							
4885	*Breweria humistrata*	Leon Co., Fla.	8/54	l, s, fl	–	0	0	0	..
4776	*Calonyction tuba*	Brunswick Co., N. C.	7/54	l, s, r	+[a]	0	0	0	..
4863	*Convolvulus arvensis*	Baltimore Co., Md.	8/54	l, s, fl, r	–	0	0	0	..
4481	*Convolvulus sepium*	Elizabeth City Co., Va.	7/54	l, s, fl	–	0	0	0	..
4734	*Ipomoea hederacea*	New Hanover Co., N. C.	7/54	l, s, r	–	0	0	0	..
4896	*Ipomoea pes-caprae*	Franklin Co., Fla.	8/54	l, s, fr, r	–	0	0	0	..
4884	*Ipomoea quamoclit*	Leon Co., Fla.	8/54	l, s, fr	–	0	0	0	..
4183	*Ipomoea sagittata*	Brunswick Co., N. C.	6/54	l, s, fl, r	–	0	0	0	0
4871	*Jacquemontia tamnifolia*	Leon Co., Fla.	8/54	l, s, fl, r	–	+	0	0	..
		CORNACEAE							
4272	*Cornus amomum*	Brunswick Co., N. C.	6/54	l, s	–	0	0	+++	..
4200	*Cornus Florida*	Wake Co., N. C.	6/54	l, s	–	0	+	0	+++
4817	*Cornus foemina*	Yates Co., N. Y.	8/54	l, s	–	0	0	++	..
4818	*Cornus stolonifera*	Yates Co., N. Y.	8/54	l, s	–	0	0	++	..
		CRASSULACEAE							
4854	*Penthorum sedoides*	Frederick Co., Md.	8/54	l, s, fl, r	–	0	0	++	..
4612	*Sedum ternatum*	Magoffin Co., Ky.	7/54	l, s, r	–	0	0	0	..
		CRUCIFERAE							
4103	*Barbarea verna*	Wake Co., N. C.	4/54	w	–	0	0	0	++
4804	*Berteroa incana*	Yates Co., N. Y.	8/54	l, s, fl, r	+[a]	0	0	0	..
4060	*Lepidium Virginicum*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			CRUCIFERAE						
4104	*Cardamine hirsuta*	Wake Co., N. C.	4/54	w	–	0	0	0	0
4679	*Rorippa islandica* (*Radicula palustris*)	Mingo Co., W. Va.	7/54	l, s, fl	–	0	0	0	..
4826	*Sisymbrium officinale*	Yates Co., N. Y.	8/54	l, s, r	–	0	0	0	..
			CUNONIACEAE						
4040A	*Weinmannia sp.*	Tingo Maria, Peru	4/54	l, s	–	++	0	++	+
4040B	*Weinmannia sp.*	Tingo Maria, Peru	4/54	b	–	+	0	+++	+
			CYPERACEAE						
4907	*Bulbostylis capillaris*	Harford Co., Md.	8/54	l, fl, r	–	0	0	0	..
4581	*Bulbostylis stenophyllus*	Brunswick Co., N. C.	7/54	l	–	0	0	0	..
4684	*Carex crinita*	Dorchester Co., Md.	7/54	l, r	–	0	0	0	..
4463	*Carex glaucescens*	Brunswick Co., N. C.	7/54	l, fl, r	–	0	0	0	..
4235	*Carex lucida*	Durham Co., N. C.	6/54	s, fl, r	–	0	0	0	0
4686	*Carex lupulina*	Dorchester Co., Md.	7/54	l, fl, r	–	0	0	0	..
4592	*Cladium jamaicense*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4568	*Cyperus lecentei*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	+++	0	0	..
4955	*Cyperus strigosus*	Leon Co., Fla.	8/54	l, fl	–	0	0	+++	..
4846	*Dulichium arundinaceum*	Wayne Co., Pa.	8/54	l, s	–	0	0	+	..
4428	*Eleocharis tuberculosa*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	+	0	0	..
4589	*Fimbristylis castanea*	Brunswick Co., N. C.	7/54	l	–	0	0	0	..
4930	*Fuirena pumila*	Sussex Co., Del.	8/54	l, fl, r	–	0	0	+	..
4462	*Fuirena spuarrosa*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4461	*Rhynchospora fascicularis*	Brunswick Co., N. C.	7/54	s, fl, r	–	0	0	0	..
4576	*Rhynchospora macrostachya*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4355	*Rhynchospora schoenoides*	Tift Co., Ga.	6/54	l, s, fl, r	–	0	0	0	..
4311	*Scirpus atrovirens* var. *Georgianus*	Chatham Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
4429	*Scirpus rubricosus* (*S. cyperinus* var. *eriophorum*)	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4591	*Scirpus robustus*	Brunswick Co., N. C.	7/54	l	–	0	0	0	..
4699	*Scirpus rubricosus*	Somerset Co., Md.	7/54	l, s, fl	–	0	0	+	..
4387	*Scleria triglomerata*	King and Queen Co., Va.	6/54	l, s, fl	–	+	0	0	..
4441	*Scleria triglomerata*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
			CRYILLACEAE						
4993	*Cliftonia monophylla*	Liberty Co., Fla.	8/54	l, s	–	0	0	+++	..
4171	*Cyrilla racemiflora*	Brunswick Co., N. C.	6/54	l, s, fl	–	+	0	+++	++
			DIAPENSIACEAE						
4412	*Pyxidanthera barbulata*	Burlington Co., N. J.	7/54	l, s, r	–	0	0	0	..
			DIOSCOREACEAE						
4127	*Dioscorea deltoides*	Karachi, Pakistan	4/54	t	+[b]	..	..	..	..
4705	*Dioscorea deltoides*	Murree, Pakistan	6/54	r	+[b]	..	..	..	..
4138	*Dioscorea quaternata*	Monongalia Co., W. Va.	5/54	r	+[b]	..	..	..	..
4006	*Dioscorea stegelmanniana*	Tingo Maria, Peru	4/54	l, s	+[a]	..	..	..	..

4706	*Dioscorea sp.*	Lima, Peru	7/54	t	–	..	..	..	..
4707	*Dioscorea sp.*	Lima, Peru	7/54	t	–	..	..	..	..
			DIPSACACEAE						
4836	*Dipsacus laciniatus*	Chemung Co., N. Y.	8/54	l, s, fr	–	0	0	0	..
4598	*Dipsacus sylvestris*	Allegany Co., Md.	7/54	l, s, fl	–	0	0	0	..
			DROSERACEAE						
4579	*Drosera intermedia*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
			EBENACEAE						
4061	*Diospyros Virginiana*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+
4126	*Diospyros Virginiana mosieri*	C. G. F.	5/54	l, s	–	0	0	0	0
			ELAEAGNACEAE						
4044	*Elaeagnus pungens*	Wake Co., N. C.	4/54	l, s	–	0	0	0	+
			EMPETRACEAE						
4903	*Ceratiola ericoides*	Franklin Co., Fla.	8/54	l, s	–	0	0	+++	..
			EQUISETACEAE						
4395	*Equisetum arvense*	Baltimore Co., Md.	7/54	l, s, r	–	0	0	0	..
4776	*Equisetum hyemale* var. *affine*	Baltimore Co., Md.	7/54	s, r	–	0	0	0	..
			ERICACEAE						
4409	*Arctostaphylos uva-ursi*	Ocean Co., N. J.	7/54	l, s, r	–	+	0	++	..
4410	*Chamaedaphne calyculata*	Burlington Co., N. J.	7/54	l, s, fl	–	++	0	+	..
4397	*Epigaea repens*	Atlantic Co., N. J.	7/54	l, s, r	–	0	0	0	..
4067	*Gaylussacia tomentosa*	McIntosh Co., Ga.	4/54	l	–	0	0	++	+
4302	*Kalmia angustifolia*	Anne Arundel Co., Md.	6/54	l, s, fr	–	0	0	++	..
4411	*Leiophyllum buxifolium*	Burlington Co., N. J.	7/54	l, s, r	–	0	0	+	..
4169	*Leiophyllum buxifolium* var. *hugeri*	Brunswick Co., N. C.	6/54	l, s, fl	–	+++	0	++	+++
4194	*Lyonia ligustrina*	Sampson Co., N. C.	6/54	l, s	–	+++	0	0	+
4177	*Lyonia mariana*	Brunswick Co., N. C.	6/54	l, s, fl, r	–	+++	0	0	+
4663	*Menziesia pilosa*	Garrett Co., Md.	7/54	l, s	–	0	0	0	..
4191	*Oxydendrum arboreum*	Sampson Co., N. C.	6/54	l, s	–	+++	0	0	+
4658	*Pyrola rotundifolia* var. *Americana*	Preston Co., W. Va.	7/54	l	–	0	0	0	..
4843	*Rhododendron Canadense*	Wayne Co., Pa.	8/54	l, s, r	–	0	0	++	..
4405	*Rhododendron viscosum*	Atlantic Co., N. J.	7/54	l, s, fl	–	0	0	+	..
4840	*Vaccinium corymbosum*	Wayne Co., Pa.	8/54	l, s, fr	–	0	0	++	..
4916	*Vaccinium macrocarpon*	Atsion, N. J.	8/54	l, s, fr, r	–	+++	0	+	..
4066	*Vaccinium myrsinites*	McIntosh Co., Ga.	4/54	l	–	0	0	++	+
4300	*Vaccinium stamineum*	Anne Arundel Co., Md.	6/54	l, s, fr	–	0	0	++	..
4192	*Vaccinium sp.*	Sampson Co., N. C.	6/54	l, s	–	+	0	0	+
4196	*Zenobia pulverulenta forma nuda*	Sampson Co., N. C.	6/54	l, s, fr	–	+++	0	++	++
			ERIOCAULACEAE						
4989	*Eriocaulon decangulare*	Liberty Co., Fla.	8/54	l, s, fr, r	–	0	0	0	..
4917	*Eriocaulon decangulare*	Atsion, N. J.	8/54	l, s, fl, r	–	0	0	0	..
			ENPHORBIACEAE						
4630	*Acalypha Virginica*	Union Co., Ky.	7/54	l, s, fl, r	–	0	0	0	..
4882	*Aleurites fordii*	Leon Co., Fla.	8/54	l, s	–	0	0	+++	..
4385A	*Aleurites Montana*	C. G. F.	7/54	l	–	0	0	++	..

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
4385B	*Aleurites Montana*	C. G. F.	7/54	s	–	0	0	0	..
4715	*Antidesma platyphyllum*	C. G. F.	7/54	l, s, fr	–	0	0	+	..
4788	*Croton Californicus*	Aguanga, Calif.	7/54	l, s	–	0	0	+++	..
4367	*Croton glandulosus*	Richmond Co., Ga.	6/54	l, s, fl, r	–	0	0	0	..
4618	*Croton monanthogynus*	Pulaski Co., Ky.	7/54	l, s, r	–	0	0	0	..
4893	*Euphorbia (Poinsettia) dentata*	Leon Co., Fla.	8/54	l, s, fl	–	0	0	0	..
4913	*Euphorbia esula*	Hammonton, N. J.	8/54	l, s, r	–	0	0	0	..
4398	*Euphorbia ipecacuanhae*	Atlantic Co., N. J.	7/54	l, s, r	–	0	0	0	..
4488	*Euphorbia maculata*	Nansemond Co., Va.	7/54	l, s, r	–	0	0	0	..
4735	*Euphorbia serpens*	New Hanover Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4898	*Stillingia aquatica*	Franklin Co., Fla.	8/54	l, s, fl	–	0	0	+++	..
4186	*Stillingia sylvatica*	Brunswick Co., N. C.	6/54	l, s, fl	–	+++	0	++	0
		FAGACEAE							
4274	*Castanea ashei*	Brunswick Co., N. C.	6/54	l, s	–	0	0	+++	..
4532	*Castanea dentata*	Buncombe Co., N. C.	7/54	l, s	–	0	0	+++	..
4290	*Castanea pumila*	Anne Arundel Co., Md.	6/54	l, s, fl	–	0	0	+++	..
4201	*Fagus grandifolia*	Wake Co., N. C.	6/54	l, s	–	0	+	0	+++
4308	*Quercus alba*	Chatham Co., N. C.	6/54	l, s	–	0	0	++	..
4929	*Quercus falcata*	Sussex Co., Del.	8/54	l, s, fr	–	0	0	+++	..
4285	*Quercus ilicifolia*	Anne Arundel Co., Md.	6/54	l, s, fr	–	0	0	++	..
4586	*Quercus marilandica*	Brunswick Co., N. C.	7/54	l, s	–	0	0	+++	..
4921	*Quercus phellos*	Charles Co., Md.	8/54	l, s	–	0	0	0	..
4401	*Quercus prinoides*	Atlantic Co., N. J.	7/54	l, s	–	0	0	++	..
4533	*Quercus prinus*	Buncombe Co., N. C.	7/54	l, s	–	0	0	++	..
4075	*Quercus pumila*	Liberty Co., Ga.	4/54	l	–	0	0	0	+
4736	*Quercus stellata* var. *margaretta*	New Hanover Co., N. C.	7/54	l, s	–	0	0	+++	..
4435	*Quercus Virginiana*	Brunswick Co., N. C.	7/54	l, s	–	0	0	++	..
		GENTIANACEAE							
4466	*Nymphoides aquatica*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4908	*Nymphoides peltata*	Elmer, N. J.	8/54	l, s, fr, r	+[a]	0	0	0	..
4850	*Sabatia angularis*	Baltimore Co., Md.	8/54	l, s, fl, r	–	0	0	0	..
4491	*Sabatia difformis*	Onslow Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4499	*Sabatia kennedyana*	Horry Co., S. C.	7/54	l, s, fl	–	0	0	0	..
4624	*Swertia Caroliniensis*	Clinton Co., Ky.	7/54	l, s, fl, r	–	0	0	0	..
		GERANIACEAE							
4137	*Geranium maculatum*	Monongalia Co., W. Va.	5/54	l, s, fl, r	–	0	0	+	+
		GRAMINEAE							
4939	*Ammophila breviligulata*	Sussex Co., Del.	8/54	l, fl, r	–	0	0	0	..
4415	*Amphicarpum purshii*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4578	*Andropogon scoparius*	Brunswick Co., N. C	7/54	l, s, r	–	++	0	0	..
4751	*Andropogon Virginicus* var. *glaucopsis*	Carteret Co., N. C.	7/54	l	–	+++	0	+	..
4910	*Aristida oligantha*	Hammonton, N. J.	8/54	l, r	–	0	+	0	..
4742	*Aristida stricta*	New Hanover Co., N. C.	7/54	l, fl, r	–	0	0	+	..
4271	*Arundinaria tecta*	Brunswick Co., N. C.	6/54	l, s	–	0	0	0	..
4446	*Arundo donax*	Brunswick Co., N. C.	7/54	l, s	–	0	+++	0	..

4721	*Axonopus compressus*	New Hanover Co., N. C.	7/54	l, fr, r	–	0	0	0	..
4696	*Cenchrus tribuloides*	Worchester Co., Md.	7/54	l, r	–	0	0	0	..
4753	*Cenchrus tribuloides*	Carteret Co., N. C.	7/54	l, fl, r	–	0	0	0	..
4432	*Ctenium aromaticum*	Brunswick Co., N. C.	7/54	l, s, fr, r	–	+	0	0	..
4722	*Cynodon dactylon*	New Hanover Co., N. C.	7/54	l, s, r	–	0	0	0	..
4567	*Digitaria sanguinalis*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4585	*Echinochloa walteri*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4487	*Eleusine indica*	Nansemond Co., Va.	7/54	l, s, fl, r	–	0	0	0	..
4240	*Elymus Virginicus*	Johnston Co., N. C.	6/54	s, r	–	0	0	0	0
4681	*Eragrostis megastachya*	Dorchester Co., Md.	7/54	l, s, r	–	0	0	0	..
4906	*Eragrostis pilosa*	Anne Arundel Co., Md.	8/54	l, r	–	0	0	0	..
4938	*Eragrostis spectabilis*	Sussex Co., Del.	8/54	l, fl, r	–	0	0	0	..
4219	*Hystrix patula*	Wake Co., N. C.	6/54	l, s, r	–	0	0	0	0
4447	*Leersia oryzoides*	Brunswick Co., N. C.	7/54	l	–	0	0	0	..
4768	*Panicum amarum*	Brunswick Co., N. C.	7/54	l, r	+°	0	0	0	..
4451	*Panicum hemitomon*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4326	*Panicum scoparium*	Wake Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
4687	*Panicum scoparium*	Dorchester Co., Md.	7/54	l, s, fl, r	–	0	0	0	..
4268	*Panicum virgatum*	Brunswick Co., N. C.	6/54	l, fl, r	+°	0	0	0	..
4256	*Paspalum dilatatum*	Wake Co., N. C.	6/54	s, r	–	0	0	0	0
4746	*Paspalum Floridanum* var. *glabratum*	Carteret Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4168	*Paspalum urvillei*	Brunswick Co., N. C.	6/54	l, s, fl	–	+	0	0	+
4733	*Phragmites communis* var. *berlandieri*	New Hanover Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4694	*Polypogon monspeliensis*	Somerset Co., Md.	7/54	s, fl, r	–	0	0	0	..
4931	*Sacciolepis striata*	Sussex Co., Del.	8/54	l, fr, r	–	0	0	0	..
4584	*Setaria magna*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4315	*Sorghum halapense*	Wake Co., N. C.	6/54	l, s, fr	–	0	0	0	..
4445	*Spartina cynosuroides*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4408	*Spartina patens*	Parkertown, Atlantic Co., N. J.	7/54	l, s, r	–	0	0	0	..
4762	*Spartina patens*	Carteret Co., N. C.	7/54	l, fl, r	–	0	0	0	..
4980	*Stenotaphrum secundatum*	Wakulla Co., Fla.	8/54	l, fl, r	–	0	0	0	..
4924	*Triodia flava*	Charles Co., Md.	8/54	l, fl, r	–	0	0	0	..
4942	*Triplasis purpurea*	Sussex Co., Del.	8/54	l, s, fl, r	–	0	0	+++	..
4255	*Tripsacum dactyloides*	Wake Co., N. C.	6/54	l, s, fl, r	–	0	0	0	0
4306	*Uniola latifolia*	Chatham Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
4920	*Uniola laxa*	Charles Co., Md.	8/54	l, r	–	0	0	0	..
4474	*Uniola paniculata*	Brunswick Co., N. C.	7/54	l, s, fr	–	0	0	0	..
4444	*Zizania aquatica*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4723	*Zizaniopsis miliacea*	New Hanover Co., N. C.	7/54	l, s	–	0	0	0	..
		GUTTIFERAE							
4430	*Ascyrum hyperidoides*	Brunswick Co., N. C.	7/54	l, s, r,	–	0	0	0	..
4342	*Hypericum densiflorum*	Leon Co., Fla.	6/54	l, s, fl	–	0	0	+	..
4559	*Hypericum densiflorum*	Macon Co., N. C.	7/54	l, s, fl	–	+	0	+	..
4633	*Hypericum dolabriforme*	Bullitt Co., Ky.	7/54	l, s, fl	–	+	0	0	..
4172	*Hypericum galioides*	Brunswick Co., N. C.	6/54	l, s, fl, r	–	+++	0	0	0
4297	*Hypericum* (*Sarothra*) *gentianoides*	Caroline Co., Va.	6/54	l, r	–	0	0	0	..
4343	*Hypericum myrtifolium*	Leon Co., Fla.	6/54	l, s, fl	–	0	0	++	..
4572	*Hypericum nudiflorum*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	+++	0	+++	..
4389	*Hypericum perforatum*	Hanover Co., Va.	7/54	l, s, fl, r	–	0	0	0	..

Accession No.	Species	Collection Source	Date Collected	Plant Part	Hemolysis test	Flavanoids	Alkaloids	Tannins	Sterols
4844	*Hypericum punctatum.*	Wayne Co., Pa.	8/54	l, s, fl	–	0	0	++	..
4613	*Hypericum spathulatum*	Clay Co., Ky.	7/54	l, s, fl	–	++	0	+	..
4456	*Hypericum tubulosum* var. *walteri*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	+	..
4915	*Hypericum Virginicum*	Atsion, N. J.	8/54	l, s, fl, r	–	0	0	++	..
		HAEMODORACEAE							
4416	*Lachnanthes tinctoria*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	..	..	..	..
4918	*Lachnanthes tinctoria*	Hammonton, N. J.	8/54	l, s, fl, r	–	0	0	0	..
		HALORAGACEAE							
4933	*Proserpinaca palustris*	Sussex Co., Del.	8/54	l, s, r	–	0	0	+++	..
		HAMAMELIDACEAE							
4424	*Hamamelis Virginiana*	Brunswick Co., N. C.	7/54	l, s	–	0	0	+	..
		HIPPOCASTANACEAE							
4147	*Aesculus octandra*	Preston Co., W. Va.	5/54	l, s	–	++	0	0	+
4505	*Aesculus octandra*	Haywood Co., N. C.	7/54	l, s	–	+	0	0	..
		HYDROCHARITACEAE							
4926	*Vallisneria Americana*	Charles Co., Md.	8/54	l, r	–	0	0	0	..
4978	*Vallisneria Americana*	Wakulla Co., Fla.	8/54	l, s, r	–	0	0	0	..
		HYDROPHYLLACEAE							
4675	*Hydrophyllum Canadense*	Harford Co., Md.	7/54	l, s, r	–	0	0	0	..
4975	*Nama corymbosum*	Wakulla Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
		IRIDACEAE							
4634	*Belamcanda Chinensis*	Bullitt Co., Ky.	7/54	l, s, r	–	0	0	0	..
		ISOETACEAE							
4864	*Isoetes riparia*	Harford Co., Md.	8/54	l, s	–	0	0	0	..
		JUGLANDACEAE							
4739	*Carya cordiformis*	New Hanover Co., N. C.	7/54	l, s	–	++	0	++	..
4198	*Carya tomentosa*	Wake Co., N. C.	6/54	l	–	+++	0	0	0
4209	*Juglans nigra*	Wake Co., N. C.	6/54	l, s	–	++	0	++	0
		JUNCACEAE							
4932	*Juncus acuminatus*	Sussex Co., Del.	8/54	l, fl, r	–	0	0	0	..
4187	*Juncus biflorus*	Brunswick Co., N. C.	6/54	s, fl, r	–	++	0	0	0
4427	*Juncus Canadensis*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4940	*Juncus Canadensis*	Sussex Co., Del.	8/54	l, fl, r	–	0	0	0	..
4426	*Juncus coriaceus*	Brunswick Co., N. C.	7/54	s, fl	–	0	0	0	..
4582	*Juncus repens*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	+++	0	0	..
4919	*Juncus scirpoides*	Charles Co., Md.	8/54	l, fl	–	0	0	0	..
		LABIATAE							
4868	*Agastache nepetoides*	Harford Co., Md.	8/54	l, s, fl, r	–	0	0	0	..
4813	*Blephilia hirsuta*	Yates Co., N. Y.	8/54	l, s, fl, r	+*	0	0	0	..
4557	*Collinsonia Canadensis*	Macon Co., N. C.	7/54	l, s, r	–	0	0	0	..
4999	*Conradina canescens*	Liberty Co., Fla.	8/54	l, s, r	–	+	0	0	..

4625	*Cunila origanoides*	Hopkins Co., Ky.	7/54	l, s, r	–	0	0	0	..
4778	*Glechoma hederacea*	Baltimore Co., Md.	7/54	l, s, r	–	0	0	0	..
4296	*Hedeoma pulegioides*	Charles Co., Md.	6/54	l, s, r	–	0	0	0	..
4874	*Hyptis mutabilis*	Leon Co., Fla.	8/54	l, s, r	–	0	0	0	..
4951	*Hyptis radiata*	Leon Co., Fla.	8/54	l, s, r	–	0	0	0	..
4878	*Leonotis nepetaefolia*	Leon Co., Fla.	8/54	l, s, fl	–	0	0	0	..
4807	*Leonurus cardiaca*	Yates Co., N. Y.	8/54	l, s, fl	+[a]	0	0	0	..
4822	*Lycopus Americanus*	Yates Co., N. Y.	8/54	l, s, r	–	0	0	0	..
4544	*Mentha piperita*	Henderson Co., N. C.	7/54	l, s, r	–	0	0	0	..
4596	*Mentha piperita*	Washington Co., Md.	7/54	l, s, fl, r	–	0	0	0	..
4319	*Monarda fistulosa*	Orange Co., N. C.	6/54	l, s, fl	–	0	0	0	..
4905	*Monarda punctata*	Anne Arundel Co., Md.	8/54	l, s, fl, r	–	0	0	0	..
4894	*Monarad punctata*	Franklin Co., Fla.	8/54	l, s, fl	–	0	0	0	..
4524	*Nepeta cataria*	Buncombe Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4599	*Nepeta cataria*	Allegany Co., Md.	7/54	l, s, fl	–	0	0	0	..
4782	*Perilla frutescens*	Harford Co., Md.	7/54	l, s, r	–	0	0	0	..
4609	*Pycanthemum clinopodioides*	Wyoming Co., W. Va.	7/54	l, s, fl, r	–	0	0	0	..
4239	*Pycanthemum flexuosum*	Durham Co., N. C.	6/54	l, s, fl	–	+	0	0	0
4293	*Pycnanthemum tenuifolium*	Charles Co., Md.	6/54	l, s, fl, r	–	+	0	0	..
4649	*Physostegia Virginiana*	Rowan Co., Ky.	7/54	l, s, fl, r	–	0	0	0	..
4362	*Salvia coccinea*	Toombs Co., N. C.	6/54	l, s, fl, r	+[a]	0	0	0	..
4802	*Salvia mellifera*	Murrieta, Calif.	7/54	w	–	+	0	0	..
4651	*Scutellaria incana*	Gilmer Co., W. Va.	7/54	l, s, fl, r	–	0	0	0	..
4273	*Scutellaria integrifolia*	Brunswick Co., N. C.	6/54	l, s, fl	–	++	0	0	..
4346	*Stachys italica*	Thomas Co., Ga.	6/54	l, s, r	–	0	0	0	..
4476	*Teucrium Canadense*	Elizabeth City Co., Va.	7/54	l, s, fl, r	–	0	0	0	..
4952	*Trichostema dichotomum*	Leon Co., Fla.	8/54	l, s, r	–	0	0	0	..
		LARDIZABALACEAE							
4521	*Akebia quinata*	Henderson Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4530	*Akebia quinata*	Buncombe Co., N. C.	7/54	l, s	–	0	0	0	..
		LAURACEAE							
4190	*Sassafras albidum*	Sampson Co., N. C.	6/54	l, s	–	++	0	0	0
		LEGUMINOSAE							
4124	*Acacia caffra*	C. G. F.	5/54	l, s	–	0	0	0	++
4158	*Acacia chloriophylla*	C. G. F.	5/54	l, s	–	++	0	0	++
4160	*Acacia concinna*	C. G. F.	5/54	l, s	+[a]	0	0	0	+
4159	*Acacia delinesn*	C. G. F.	5/54	l, s	–	0	0	0	0
4161	*Acacia farnesiana*	C. G. F.	5/54	l, s	–	0	+	0	+
4162	*Acacia galpinii*	C. G. F.	5/54	l, s	+[a]	0	0	0	+
4163	*Acacia koa*	C. G. F.	5/54	l, s, fr	–	++	0	0	++
4164	*Acacia laeta*	C. G. F.	5/54	l, s	+[a]	0	0	0	++
4165	*Acadia pallens*	C. G. F.	5/54	l, s	–	+++	0	0	+++
4729	*Aeschynomone indica*	New Hanover Co., N. C.	7/54	l, s, fl, r	–	0	0	0	
4229A	*Albizzia adinocephala*	C. G. F.	6/54	l	+[a]	0	0	0	+++
4229B	*Albizzia adinocephala*	C. G. F.	6/54	s	+[a]	0	0	0	0
4381	*Albizzia caribaea*	C. G. F.	7/54	l, s	–	0	++	0	..
4382	*Albizzia chevalieri*	C. G. F.	7/54	l, s	–	0	0	0	..
4225	*Albizzia Chinensis*	C. G. F.	6/54	l, s	–	0	0	++	++
4228A	*Albizzia corbissieri*	C. G. F.	6/54	l	+[a]	0	0	0	++

Accession No.	Species	Collection Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
		LEGUMINOSAE							
4228B	*Albizzia corbissieri*	C. G. F.	6/54	s	+[a]	0	0	0	0
4230A	*Albizzia falcata*	C. G. F.	6/54	l	+[a]	0	0	++	++
4230B	*Albizzia falcata*	C. G. F.	6/54	s	+[a]	0	0	0	++
4312	*Albizzia julibrissin*	Chatham Co., N. C.	6/54	l, s	+[a]	0	0	0	..
4231A	*Albizzia lucida*	C. G. F.	6/54	l	+[a]	0	+	0	+
4231B	*Albizzia lucida*	C. G. F.	6/54	s	+[a]	0	+++	0	+
4232A	*Albizzia mollis*	C. G. F.	6/54	l	+[a]	0	0	0	0
4232B	*Albizzia mollis*	C. G. F.	6/54	s	+[a]	0	0	0	0
4233A	*Albizzia richardiana*	C. G. F.	6/54	l	−	0	++	0	++
4233B	*Albizzia richardiana*	C. G. F.	6/54	s	+[a]	0	0	0	+
4226	*Albizzia sassa*	C. G. F.	6/54	l, s	−	0	0	0	0
4227	*Albizzia zygia*	C. G. F.	6/54	l, s	−	0	0	0	0
4175	*Amorpha cyanostachya*	Brunswick Co., N. C.	6/54	l, s, fl	−	++	0	0	++
4925	*Amorpha cf. fruticosa*	Charles Co., Md.	8/54	l, s, fr	−	0	0	+++	..
4423	*Amorpha herbacea*	Brunswick Co., N. C.	7/54	l, s, fl	−	0	0	0	..
4795	*Astragalus pomonensis*	Dripping Springs, Calif.	7/54	l	−	0	0	0	..
4700	*Baptisia alba*	Accomac, Va.	7/54	l, s, fr, r	−	0	+++	0	..
4372	*Baptisia cinerea*	Moore Co., N. C.	6/54	l, s	−	++	0	0	..
4339	*Baptisia psammophila*	Leon Co., Fla.	6/54	l, s, fr, r	−	0	++	0	..
4242	*Baptisia tinctoria*	Wake Co., N. C.	6/54	l, s, fl, r	−	+	+	0	0
4284	*Baptisia tinctoria*	Alexandria, Va.	6/54	l, s, fl	−	+	++	0	..
4482	*Cassia fasciculata*	Elizabeth City Co., Va.	7/54	l, s, r	−	0	0	0	..
4888	*Cassia cf. littoralis*	Leon Co., Fla.	8/54	l, s	−	0	0	+++	..
4848	*Cassia Marilandica*	Baltimore Co., Md.	8/54	l, s, fl, r	−	0	0	0	..
4889	*Cassia cf. multipinnata*	Leon Co., Fla.	8/54	l, s, r	−	0	+	0	..
4569	*Cassia nictitans*	Brunswick Co., N. C.	7/54	l, s, r	−	+	0	0	..
4879	*Cassia occidentalis*	Leon Co., Fla.	8/54	l, s, fr	−	0	0	0	..
4338	*Cassia tora*	Leon Co., Fla.	6/54	l, s, r	−	0	0	0	..
4587	*Cassia tora*	Brunswick Co., N. C.	7/54	l, s, fl	−	0	0	0	..
4760	*Centrosema Virginianum*	Carteret Co., N. C.	7/54	l, s, fr	−	0	0	0	..
4725	*Clitoria mariana*	New Hanover Co., N. C.	7/54	l, s, r	−	0	0	0	..
4849	*Coronilla varia*	Baltimore Co., Md.	8/54	l, s, r	−	0	+	0	..
4957	*Crotalaria angulata*	Leon Co., Fla.	8/54	l, s, fr, r	−	0	0	0	..
4945	*Crotalaria cf. mucronata*	Leon Co., Fla.	8/54	l, s, fr, r	−	0	0	++	..
4872	*Crotalaria spectabilis*	Leon Co., Fla.	8/54	l, s, r	−	0	0	0	..
4034	*Crotalaria sp.*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	++
4356	*Daubentonia punicea*	Irwin Co., Ga.	6/54	l, s, fr	+[a]	0	0	0	..
4838	*Desmodium Canadense*	Bradford Co., Pa.	8/54	l, s, fl	−	0	0	0	..
4571	*Desmodium ciliare*	Brunswick Co., N. C.	7/54	l, s, fl, r	−	+	0	0	..
4964	*Desmodium lineatum*	Leon Co., Fla.	8/54	l, s, r	−	0	0	++	..
4337	*Desmodium paniculatum*	Thomas Co., Ga.	6/54	l, s, r	−	0	0	0	0
4727	*Desmodium tenuifolium*	New Hanover Co., N. C.	7/54	l, s, fl, r	−	0	0	0	..
4728	*Desmodium tortuosum*	New Hanover Co., N. C.	7/54	l, s, r	+[a]	0	0	0	..
4965	*Desmodium virdiflorum*	Leon Co., Fla.	8/54	l, s, fl, r	−	0	0	+++	..
4977	*Galactia mollis*	Wakulla Co., Fla.	8/54	l, s, fl	−	0	0	+	..
4371	*Galactia regularis*	Moore Co., N. C.	6/54	l, s, fl	−	0	0	0	..

4880	*Gleditsia triacanthos*	Leon Co., Fla.	8/54	l, s, fr	–	0	0	++	..
4053	*Glottidium vesicarium*	McIntosh Co., Ga.	4/54	l	–	0	0	0	++
4644	*Gymnocladus dioica*	Mason Co., Ky.	7/54	l, s	–	0	0	0	..
4179	*Indigofera Caroliniana*	Brunswick Co., N. C.	6/54	l, s	–	0	0	0	+
4500	*Lathyrus latifolius*	Spartenberg Co., S. C.	7/54	l, s, fl, fr	–	0	0	0	..
4954	*Lespedeza angustifolia*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	+++	..
4759	*Lespedeza bicolor*	Carteret Co., N. C.	7/54	l	–	0	0	0	..
4373	*Lespedeza capitata*	Scotland Co., N. C.	6/54	l, s, fl	–	0	0	+	..
4413	*Lespedeza capitata*	Camden Co., N. J.	7/54	l, s, fl	–	0	0	+	..
4251	*Lespedeza cuneata*	Wake Co., N. C.	6/54	l, s	–	0	0	0	0
4741	*Lespedeza hirta* var. *appressipilis*	New Hanover Co., N. C.	7/54	l, s, fl, r	–	0	0	++	..
4205	*Lespedeza procumbens*	Wake Co., N. C.	6/54	l, s, r	–	0	0	0	+
4963	*Lespedeza repens*	Leon Co., Fla.	8/54	l, s	–	0	0	+++	..
4443	*Lespedeza stuevei*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	+++	..
4212	*Lespedeza Virginica*	Wake Co., N. C.	6/54	l, s, r	–	+	0	0	0
4811	*Lotus corniculatus*	Yates Co., N. Y.	8/54	l, s, fl	–	0	0	0	..
4800	*Lupinus excubitus*	Dripping Springs, Calif.	7/54	w	–	0	+++	0	..
4077	*Lupinus nuttallii*	Bryan Co., Ga.	4/54	l, s	–	0	+++	0	+
4851	*Medicago lupulina*	Baltimore Co., Md.	8/54	l, s, fl, r	+[a]	0	0	0	..
4852	*Medicago sativa*	Carroll Co., Md.	8/54	l, s, fl, r	+[a]	0	0	0	..
4207	*Melilotus alba*	Wake Co., N. C.	6/54	l, s, fl, r	–	0	0	0	0
4639	*Petalostemum candidum*	Bullitt Co., Ky.	7/54	l, s, fl, r	–	0	0	0	..
4901	*Petalostemum feayi*	Franklin Co., Fla.	8/54	l, s, fl	–	0	0	+	..
4887	*Petalostemum pinnatum*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
4997	*Pitcheria galactioides*	Liberty Co., Fla.	8/54	l, s, r	–	+	0	0	..
4363	*Psoralea canescens*	Toombs Co., Ga.	6/54	l, s, fl, r	–	0	0	0	..
4238	*Psoralea psoralioides*	Durham Co., N. C.	6/54	l, s, fl	+[a]	0	0	0	0
4614	*Psoralea psoralioides* var. *eglandulosa*	Laurel Co., Ky.	7/54	l, s, fl	–	0	0	0	..
4047	*Pueraria lobata*	Wake Co., N. C.	4/54	l, s	–	0	0	0	..
4099	*Pueraria lobata*	Clarke Co., Ga.	4/54	l	–	0	0	0	+++
4493	*Rhynchosia difformis*	Pender Co., N. C.	7/54	l, s, fl, r	–	+	0	0	..
4959	*Rhynchosia simplicifolia*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
4467	*Robinia cf. nana*	Brunswick Co., N. C.	7/54	l, s	–	0	0	0	..
4522	*Schrankia microphylla*	Henderson Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4726	*Strophostyles helvola*	New Hanover Co., N. C.	7/54	l, s, r	–	0	0	0	..
4958	*Stylosanthes biflora*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
4580	*Tephrosia Florida*	Brunswick Co., N. C.	7/54	l, s, fr, r	–	++	0	0	..
4211	*Tephrosia Virginiana*	Wake Co., N. C.	6/54	l, s	–	++	+	0	0
4042	*Tephrosia sp.*	Tingo Maria, Peru	4/54	l, s	–	+	0	0	+
4101	*Vicia villosa*	Wake Co., N. C.	4/54	l, s	–	0	0	0	..
4724	*Vigna luteola*	New Hanover Co., N. C.	7/54	l, s, fr	–	0	0	0	..
4774	*Vigna sinensis*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4184	*Zornia bracteata*	Brunswick Co., N. C.	6/54	l, s, r	–	0	0	0	0
			LEMNACEAE						
4781	*Spirodela polyrhiza* and *Lemna sp.*	Harford Co., Md.	7/54	1	–	0	0	0	..
			LENTIBULARIACEAE						
4909	*Utricularia sp.*	Elmer, N. J.	8/54	1	–	0	0	0	..

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			LILIACEAE						
4351	*Aletris aurea*	Tift Co., Ga.	6/54	l, s, fl, r	+[a]	0	0	0	..
4721	*Asparagus racemosus*	C. G. F.	7/54	l, s	+[a]	0	0	0	..
4653	*Clintonia umbellulata*	Monongalia Co., W. Va.	7/54	l, s, r	+[b]	0	0	0	..
4654	*Disporum lanuginosum*	Monongalia Co., W. Va.	7/54	l, s, fr	–	0	0	0	..
4142	*Erythronium Americanum*	Monongalia Co., W. Va.	5/54	l, s	–	0	+	0	++
4563	*Lilium superbum*	Macon Co., N. C.	7/54	l, s, fl, r	–	0	++	0	..
4855	*Medeola Virginiana*	Frederick Co., Md.	8/54	l, s, fr, r	–	0	0	0	..
4357	*Melanthium Virginicum*	Irwin Co., Ga.	6/54	l, s, fl, r	–	0	++	0	..
4048	*Polygonatum biflorum*	Wake Co., N. C.	4/54	l, s	–	0	0	0	0
4049	*Smilacina racemosa*	Wake Co., N. C.	4/54	l, s	–	0	0	0	+
4088	*Smilax rotundifolia*	Clarke Co., Ga.	4/54	l, s	–	0	0	0	0
4979	*Smilax sp.*	Leon Co., Fla.	8/54	t	–	0	0	+	..
4678	*Trillium flexipes*	Harford Co., Md.	7/54	l, s, fr	+[b]	0	0	0	..
4146	*Trillium grandiflorum*	Preston Co., W. Va.	5/54	l, s, r	+[a]	0	0	0	0
4988	*Zigadenus glaberrimus*	Liberty Co., Fla.	8/54	l, s, fl, r	–	0	0	+	..
			LINACEAE						
4704	*Linum Floridanum*	Onslow Co., N. C.	7/54	l, s, fl, r	–	0	0	+	..
4388	*Linum Virginianum*	Caroline Co., Va.	6/54	l, s, fl, r	–	0	0	0	..
			LOBELIACEAE						
4695	*Lobelia cardinalis*	Somerset Co., Md.	7/54	l, s, fl, r	–	0	++	0	..
4985	*Lobelia glandulifera*	Liberty Co., Fla.	8/54	l, s, r	–	0	0	0	..
			LOGANIACEAE						
4454	*Polypremum procumbens*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
			LYCOPODIACEAE						
4761	*Lycopodium alopecuroides*	Carteret Co., N. C.	7/54	w	–	0	++	0	..
4314	*Lycopodium complanatum* var. *flabelliforme*	Chatham Co., N. C.	6/54	w	–	0	+++	0	..
4404	*Lycopodium inundatum* var. *bigelovii*	Atlantic Co., N. J.	7/54	w, r	–	0	+++	0	..
4657	*Lycopodium lucidulum*	Preston Co., W. Va.	7/54	w	–	0	+++	0	..
			LYTHRACEAE						
4934	*Decodon verticillatus*	Sussex Co., Del.	8/54	l, s, fl	–	0	0	+++	..
4449	*Lagerstroemia indica*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4972	*Lythrum lineare*	Wakulla Co., Fla.	8/54	l, s	–	0	0	+++	..
			MAGNOLIACEAE						
4093	*Liriodendron tulipifera*	Clarke Co., Ga.	4/54	l	–	0	+	0	+
4623	*Magnolia acuminata*	Wayne Co., Ky.	7/54	l, s	–	0	++	0	..
4511	*Magnolia fraseri*	Haywood Co., N. C.	7/54	l, s	–	0	0	0	..
4222	*Magnolia tripetala*	Wake Co., N. C.	6/54	l, s, fr	–	0	0	0	0
4080	*Magnolia Virginiana*	Bulloch Co., Ga.	4/54	l	–	0	0	0	0
			MALVACEAE						
4174	*Abutilon theophrasti*	Baltimore Co., Md.	6/54	l, s, r	–	..	..	..	..
4354	*Hibiscus aculeatus*	Tift Co., Ga.	6/54	l, s, fr, r	–	0	0	0	..
4359	*Hibiscus militaris*	Irwin Co., Ga.	6/54	l, s, fl, r	–	0	0	0	..

4631	*Hibiscus militaris*	Daviess Co., Ky.	7/54	l, s	−	0	0	0	..
4275	*Hibiscus moscheutos*	Chatham Co., N. C.	6/54	l, s	−	0	0	0	..
4701	*Hibiscus trionum*	Somerset Co., Md.[b]	7/54	l, s, fl, r	−	0	0	0	..
4477	*Kosteletzkya Virginica*	Elizabeth City Co., Va.	7/54	l, s, r	−	0	0	0	..
4600	*Malva moschata*	Garrett Co., Md.	7/54	l, s, fl, r	+[a]	0	0	0	..
4805	*Malva rotundifolia*	Yates Co., N. Y.	8/54	l, s, fl, r	−	0	0	0	..
4370	*Sida rhombifolia*	Scotland Co., N. C.	6/54	l, s, fl	−	0	0	0	..
4783	*Sida spinosa*	Baltimore Co., Md.	7/54	l, s, fl, r	−	0	0	0	..
		MELASTOMATACEAE							
4023	*Clidemia capitellata*	Tingo Maria, Peru	4/54	l, s	−	0	0	++	+++
4038	*Leandra dichotoma*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	+
4037	*Miconia clathrantha*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	++
4336	*Rhexia alifanus*	Leon Co., Fla.	6/54	l, s, fl, fr, r	−	0	0	0	..
4335	*Rhexia cubensis*	Leon Co., Fla	6/54	l, s, fl, r	−	0	0	0	..
4358	*Rhexia lutea*	Tift Co., Ga.	6/54	l, s, fl, r	−	0	0	0	..
4298	*Rhexia mariana*	Hanover Co., Va.	6/54	l, s, fl	−	0	0	0	..
4574	*Rhexia mariana*	Brunswick Co., N. C.	7/54	l, s, fl, r	−	0	0	0	..
		MENISPERMACEAE							
4091	*Cocculus Carolinus*	Clarke Co., Ga.	4/54	l	−	0	+	0	+
4140	*Menispermum Canadense*	Monongalia Co., W. Va.	5/54	l, s	−	0	+++	0	0
		MORACEAE							
4720	*Artocarpus hypargyrea*	C. G. F.	7/54	l, s	−	0	0	0	..
4095	*Broussonetia papyrifera*	Clarke Co., Ga.	4/54	l	−	0	0	0	0
4289	*Broussonetia papyrifera*	Baltimore Co., Md.	6/54	l, s	−	0	0	0	..
4116	*Ficus bonplandiana*	C. G. F.	5/54	l, s, fr	−	0	0	0	0
4113	*Ficus callosa*	C. G. F.	4/54	l, s	−	0	0	0	++
4108	*Ficus carica*	C. G. F.	4/54	l, s	−	0	0	0	++
4111	*Ficus cotinifolia*	C. G. F.	4/54	l, s	−	0	0	0	0
4118	*Ficus iteophylla*	C. G. F.	5/54	l, s	−	0	0	0	0
4106	*Ficus kerstingii*	C. G. F.	4/54	l, s	−	0	0	0	+
4115	*Ficus lucescens*	C. G. F.	4/54	l, s, fr	−	0	0	0	+
4112	*Ficus nymphaeaefolia*	C. G. F.	4/54	l, s	−	0	0	0	+
4114	*Ficus odorata*	C. G. F.	4/54	l, s	−	0	0	0	+
4117	*Ficus petiolaris*	C. G. F.	5/54	l, s	−	0	0	0	++
4105	*Ficus payapa*	C. G. F.	4/54	l, s, fr, r	−	0	0	0	0
4119	*Ficus radula*	C. G. F.	5/54	l, s	−	0	0	0	0
4120	*Ficus radulina*	C. G. F.	5/54	l, s	−	0	0	0	0
4109	*Ficus trachyphylla*	C. G. F.	4/54	l, s, fr, r	−	0	0	0	+
4107	*Ficus tsiela*	C. G. F.	4/54	l, s, fr, r	−	0	0	0	+
4110	*Ficus urbaniana*	C. G. F.	4/54	l, s	−	0	0	0	+
4157	*Ficus urbaniana*	C. G. F.	5/54	l, s	−	0	0	0	0
4121	*Ficus urceolaris*	C. G. F.	5/54	l, s	−	0	0	0	0
4254	*Humulus Japonicus*	Johnston Co., N. C.	6/54	l, s	−	0	0	0	+
4288	*Maclura pomifera*	Baltimore Co., Md.	6/54	l, s	−	0	0	0	..
4241	*Maclura pomifera*	Wake Co., N. C.	6/54	l, s	−	0	0	0	0
4046	*Morus alba*	Wake Co., N. C.	4/54	l, s	−	0	0	0	++
		MYRICACEAE							
4283	*Comptonia peregrina*	Anne Arundel Co., Md.	6/54	l, s	−	0	0	++	..
4995	*Myrica Carolinensis* (*Pensylvanica*)	Liberty Co., Fla.	8/54	l, s, fr	−	0	0	+++	..
4740	*Myrica pusilla*	New Hanover Co., N. C.	7/54	l, s	−	0	0	+++	..

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			MYRTACEAE						
4122	*Eucalyptus Algeriensis*	C. G. F.	5/54	l, s	–	0	0	0	0
			NYCTAGINACEAE						
4494	*Mirabilis jalapa*	Craven Co., N. C.	7/54	l, s, fl	–	0	+	0	..
4912	*Mirabilis nyctaginea*	Hammonton, N. J.	8/54	l, s, fl, r	–	0	+	0	..
			NYMPHAEACEAE						
4947	*Nelumbo lutea*	Leon Co., Fla.	8/54	l, s, fl, fr	–	0	0	0	..
4987	*Nuphar advena*	Liberty Co., Fla.	8/54	l, s, fr, r	–	0	+	0	..
4299	*Nymphaea odorata*	Anne Arundel Co., Md.	6/54	l, s, r	–	0	0	0	..
4465	*Nymphaea odorata*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
			NYSSACEAE						
4489	*Nyssa aquatica*	Bertie Co., N. C.	7/54	l, s	–	0	0	0	..
4173	*Nyssa sylvatica* var. *biflora*	Brunswick Co., N. C.	6/54	l, s	–	+	0	0	+++
			OLEACEAE						
4050	*Chionanthus Virginicus*	Hancock Co., Ga.	4/54	l	–	0	0	0	+++
4203	*Fraxinus Americana*	Wake Co., N. C.	6/54	l, s	–	++	+	0	++
4176	*Fraxinus Caroliniana*	Brunswick Co., N. C.	6/54	l, s	–	0	0	0	++
4096	*Jasminum humile*	Clarke Co., Ga.	4/54	l	–	0	0	0	++
			ONAGRACEAE						
4664	*Epilobium angustifolium*	Garrett Co., Md.	7/54	l, s, fl, r	–	0	0	+++	..
4821	*Epilobium hirsutum*	Yates Co., N. Y.	8/54	l, s, fl, r	–	0	0	+++	..
4182	*Gaura biennis*	Brunswick Co., N. C.	6/54	l, s, r	–	+++	0	++	0
4961	*Gaura filipes*	Leon Co., Fla.	8/54	l, s, r	–	0	0	0	0 ..
4188	*Jussiaea michauxiana*	New Hanover Co., N. C.	7/54	l, s, r	–	+	0	++	0
4407	*Kneiffia linearis* (*Oenothera fruticosa* var. *linearis*)	Atlantic Co., N. J.	7/54	l, s, fl, r	–	0	0	++	..
4352	*Ludwigia alternifolia*	Benhill Co., Ga.	6/54	l, s, r	–	0	0	+++	..
4566	*Ludwigia linearis*	Brunswick Co., N. C.	7/54	l, s	–	+	0	+++	..
4986	*Ludwigia pilosa*	Liberty Co., Fla.	8/54	l, s, fl, r	–	0	0	+++	..
4459	*Ludwigia suffruticosa*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	++	..
4353	*Ludwigia virgata*	Benhill Co., Ga.	6/54	l, s, fl, r	–	0	0	+++	..
4166	*Oenothera biennis*	Brunwsick Co., N. C.	6/54	l, s, r	–	+	0	+	+
4922	*Trapa natans*	Charles Co., Md.	8/54	fr	–	0	0	0	..
			OPHIOGLOSSACEAE						
4862	*Botrychium Virginianum*	Frederick Co., Md.	8/54	l, s	–	0	0	0	..
			OROBANCHACEAE						
4870	*Conopholis Americana*	Harford Co., Md.	8/54	s, fr	–	0	0	0	..
			OSMUNDACEAE						
4603	*Osmunda cinnamonea*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+
4856	*Osmunda claytoniana*	Frederick Co., Md.	8/54	l, s, r	–	0	0	0	..
4072	*Osmunda regalis*	McIntosh Co., Ga.	4/54	l	–	0	0	0	0
			OXALIDACEAE						
4785	*Oxalis Europaea f. cymoza*	Baltimore Co., Md.	7/54	l, s, r	–	0	0	0	..
4086	*Osalic stricta*	Clarke Co., Ga.	4/54	l, s	+[a]	0	0	0	0

			PALMAE						
4710	*Acoeloraphe arborescens*	C. G. F.	7/54	l, s	–	0	0	+++	..
4224	*Aeria attenuata*	C. G. F.	6/54	l, s, fr	–	0	0	0	0
4718	*Areca cathcu*	C. G. F.	7/54	l, s, fr	–	0	++	0	..
4380	*Arecastrum romanzoffianum*	C. G. F.	7/54	l, s, fr	–	0	0	0	..
4349	*Butia capitata*	Irwin Co., Ga.	6/54	s, fr	–	0	0	0	..
4973	*Sabal palmetto*	Wakulla Co., Fla.	8/54	l, s	–	0	0	+	..
4886	*Serenoa repens*	Leon Co., Fla.	8/54	l, s	–	0	0	+++	..
			PAPAVERACEAE						
4133	*Chelidonium majus*	Washington Co., Md.	5/54	l, s	–	0	++	0	0
4859	*Sanguinaria Canadensis*	Frederick Co., Md.	8/54	l, s, r	–	0	+++	0	..
			PASSIFLORACEAE						
4330	*Passiflora incarnata*	Wake Co., N. C.	6/54	l, s, fr, r	–	0	+	0	..
			PHYTOLACCACEAE						
4069	*Phytolacca Americana*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+
4276	*Phytolacca Americana*	Baltimore Co., Md.	6/54	l, s	+*	0	0	0	..
			PINACEAE						
4403	*Chamaecyparis thyoides*	Atlantic Co., N. J.	7/54	l, s	–	0	0	0	..
4549	*Picea abies*	Macon Co., N. C.	7/54	l, s	–	0	0	+	..
4998	*Pinus caribaea*	Liberty Co., Fla.	8/54	l, s	–	0	0	+	..
4440	*Pinus serotina*	Brunswick Co., N. C.	7/54	l, s, fr	–	0	0	0	..
4514	*Pinus strobus*	Haywood Co., N. C.	7/54	l, s	–	0	0	0	..
4452	*Taxodium ascendens*	Brunswick Co., N. C.	7/54	l, s, fr	–	+	0	+	..
4550	*Thuja occidentalis*	Transylvania Co., N. C.	7/54	l, s	–	0	0	++	..
4502	*Tsuga Canadensis*	Haywood Co., N. C.	7/54	l, s	–	0	0	0	..
4662	*Tsuga Canadensis*	Garrett Co., Md.	7/54	l, s	–	0	0	0	..
			PIPERACEAE						
4001	*Peperomia sp.*	Tingo Maria, Peru	4/54	l, r	–	0	0	0	+
4003	*Piper sp.*	Tingo Maria, Peru	4/54	l, s	–	0	0	0	+++
			PLANTAGINACEAE						
4259	*Plantago aristata*	Wake Co., N. C.	6/54	l, s, fl	–	0	0	0	..
4396	*Plantago aristata*	Baltimore Co., Md.	7/54	l, s, fr, r	–	0	0	0	..
4055	*Plantago Virginica*	McIntosh Co., Ga.	4/54	l	–	0	0	0	++
			PLATANACEAE						
4210	*Platanus occidentalis*	Wake Co., N. C.	6/54	l, s	–	0	0	0	+
			PLUMBAGINACEAE						
4773	*Limonium Carolinianum*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	+	..
			POLEMONIACEAE						
4562	*Phlox Carolina* var. *triflora*	Macon Co., N. C.	7/54	l, s, fl, r	–	+	0	0	..
4144	*Phlox divaricata* (var. *Canadensis*)	Monongalia Co., W. Va.	5/54	l, s, fl, r	–	0	0	0	+
4316	*Phlox maculata*	Durham Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
			POLYGALACEAE						
4914	*Polygala cruciata*	Atsion, N. J.	8/54	l, s, fl, r	–	0	0	0	..
4181	*Polygala lutea*	Brunswick Co., N. C.	6/54	l, s, r, fl	–	++	0	0	+

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis Test	Flavonoids	Alkaloids	Tannins	Sterols
4390	*Polygala mariana*	Caroline Co., Va.	7/54	l, s	+*	0	0	0	..
4434	*Polygala ramosa*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
		POLYGONACEAE							
4798	*Eriogonum gracile*	Dripping Springs, Calif.	7/54	w	–	+	0	0	..
4360	*Eriogonum tomentosum*	Emanuel Co., N. C.	6/54	l, s, fl, r	–	0	0	++	..
4820	*Fagopyrum esculentum (F. sagittatum)*	Yates Co., N. C.	8/54	l, s, fl, r	–	0	0	0	..
4368	*Polygonella Americana*	Richmond Co., Ga.	6/54	l, s, fl, r	–	0	0	0	..
4984	*Polygonella gracilis*	Liberty Co., Fla.	8/54	s, r	–	0	0	++	..
4730	*Polygonum arifolium*	New Hanover Co., N. C.	7/54	l, s	–	0	0	+	..
4869	*Polygonum cuspidatum*	Harford Co., Md.	8/54	l, s, fl	–	0	0	+	..
4318	*Polygonum Pensylvanicum*	Wake Co., N. C.	6/54	l, s	–	0	0	0	..
4899	*Thysanella fimbriata*	Franklin Co., Fla.	8/54	l, s, fl, r	–	0	0	++	..
4861	*Tovara Virginiana*	Frederick Co., Md.	8/54	l, s, fl, r	–	0	0	0	..
		POLYPODIACEAE							
4857	*Adiantum pedatum*	Frederick Co., Md.	8/54	l, s, r	–	0	0	+	..
4669	*Athyrium thelypteroides*	Baltimore Co., Md.	7/54	l, s	–	0	0	0	..
4548	*Dennstaedtia punctilobula*	Transylvania Co., N. C.	7/54	l, s, r	–	0	0	0	..
4858	*Dryopteris hexagonoptera*	Frederick Co., Md.	8/54	l, s, r	–	0	0	0	..
4677	*Dryopteris marginalis*	Harford Co., Md.	7/54	l, s, r	–	0	0	0	..
4213	*Dryopteris noveboracensis*	Wake Co., N. C.	6/54	l	–	0	+	0	0
4536	*Onoclea sensibilis*	Buncombe Co., N. C.	7/54	l, s	–	0	0	0	..
4655	*Polypodium Virginianum*	Monongalia Co., W. Va.	7/54	l, s, r	–	0	0	0	..
4438	*Woodwardia areolata*	Brunswick Co., N. C.	7/54	l, s	–	0	0	0	..
		PONTEDERIACEAE							
4071	*Pontederia cordata*	McIntosh Co., Ga.	4/54	l	–	0	0	0	0
		PORTULACACEAE							
4731	*Portulaca oleracea*	New Hanover Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4784	*Portulaca oleracea*	Baltimore Co., Md.	7/54	l, s, fl, r	–	0	0	0	..
		POTAMOGETONACEAE							
4927	*Potamogeton cf. perfoliatus*	Charles Co., Md.	8/54	l, s, fl	–	0	0	0	..
		PRIMULACEAE							
4601	*Lysimachia quadrifolia*	Tucker Co., W. Va.	7/54	l, s, r	–	+	0	0	..
4564	*Lysimachia quadrifolia*	Macon Co., N. C.	7/54	l, s, fl, r	–	+	0	0	..
4867	*Lysimachia vulgaris*	Harford Co., Md.	8/54	l, s, fl, r	–	0	0	0	..
		RANUNCULACEAE							
4606	*Anemone Virginiana*	Raleigh Co., W. Va.	7/54	l, s, fl, r	+*	0	0	0	..
4129	*Anemonella thalictroides*	Washington Co., Md.	5/54	l, s	–	0	+	0	++
4139	*Cimicifuga racemosa*	Monongalia Co., W. Va.	5/54	l, s, r	–	0	0	0	0
4141	*Clematis Virginiana*	Monongalia Co., W. Va.	5/54	l, s	–	0	0	0	+
4149	*Hepatica acutiloba*	Preston Co., W. Va.	5/54	l, s, r	–	0	0	0	+
4801	*Paeonia brownii*	Dripping Springs, Calif.	7/54	w	–	+	0	+++	..
4406	*Tholictrum polygamum*	Atlantic Co., N. J.	7/54	l, fl, r	–	0	++	0	..
4542	*Xanthorrhiza simplicissima*	Henderson Co., N. C.	7/54	l, s, r	–	0	+	0	..

			RHAMNACEAE						
4749	*Berchemia scandens*	Carteret Co., N. C.	7/54	l, s	–	+	0	+	..
4223	*Ceanothus Americanus*	Wake Co., N. C.	6/54	l, s, fl	–	0	++	++	0
4824	*Rhamnus catharticus*	Yates Co., N. Y.	8/54	l, s, fr	–	0	0	0	..
4622	*Rhamnus lanceolatus*	Wayne Co., Ky.	7/54	l, s	–	0	0	0	..
			ROSACEAE						
4792	*Adenostoma sparsifolium*	Dripping Springs, Calif.	7/54	l	–	0	0	++	..
4531	*Agrimonia gryposepala*	Buncombe Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4309	*Agrimonia parviflora*	Chatham Co., N. C.	6/54	l, s, r	–	0	0	0	..
4195	*Aronia arbutifolia*	Sampson Co., N. C.	6/54	l, s	–	+	0	0	0
4603	*Aruncus dioicus*	Clay Co., W. Va.	7/54	l, s, fl, r	–	0	0	0	..
4670	*Chaenomeles lagenaria*	Baltimore Co., Md.	7/54	l, s, fr	–	0	0	0	..
4996	*Chrysobalanus oblongifolius*	Liberty Co., Fla.	8/54	l, s, fr	–	0	0	+++	..
4815	*Geum aleppicum* var. *strictum*	Yates Co., N. Y.	8/54	l, s, fl, r	–	0	0	++	..
4626	*Gillenia stipulata*	Hopkins Co., Ky.	7/54	l, s, fl, r	–	0	0	+	..
4602	*Gillenia trifoliata*	Randolph Co., W. Va.	7/54	l, s, fl	–	0	0	0	..
4394	*Physocarpus opulifolius*	Baltimore Co., Md.	7/54	l, s, fl	–	0	0	0	..
4812	*Potentilla recta*	Yates Co., N. Y.	8/54	l, s, fl, r	–	0	0	+	..
4765	*Prunus ilicifolia*	Aguanga, Calif.	8/54	l	–	0	0	+++	..
4944	*Prunus maritima*	Sussex Co., Del.	8/54	l, s	–	0	0	+++	..
4560	*Prunus Pensylvanica*	Macon Co., N. C.	7/54	l, s	–	0	0	+	..
4823	*Prunus Virginiana*	Yates Co., N. Y.	8/54	l. fr, r	–	0	0	0	..
4145	*Pyrus (Malus) coronaria*	Taylor Co., W. Va.	5/54	l. s	–	+++	0	++	++
4943	*Pyrus (Malus) sp.*	Sussex Co., Del.	8/54	l, s	–	0	0	+++	..
4479	*Rosa Carolina*	Elizabeth City Co., Va.	7/54	l, s	–	+	0	+++	..
4327	*Rubus argutus*	Wake Co., N. C.	6/54	l, s, fl	–	0	0	0	..
4767	*Rubus cuneifolius*	Carteret Co., N. C.	7/54	l, s	–	0	0	++	..
4523	*Rubus idaeus* var. *Canadensis*	Buncombe Co., N. C.	7/54	l, s	–	0	0	0	..
4832	*Rubus idaeus* var. *strigosus*	Cayuta Lake, N. Y.	8/54	l, s	–	0	0	0	..
4508	*Rubus odoratus*	Haywood Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4661	*Rubus odoratus*	Garrett Co., Md.	7/54	l, s	–	0	0	0	..
4667	*Rubus phoenicolasius*	Baltimore Co., Md.	7/54	l, s, fl	–	0	0	+	..
4780	*Sanguisorba Canadensis*	Baltimore Co., Md.	7/54	l, s	–	0	0	+++	..
4837	*Sorbaria sorbifolia*	Bradford Co., Pa.	8/54	l, s, fl, r	–	0	0	0	..
4703	*Spiraea tomentosa*	Wicomico Co., Md.	7/54	l, s, r	–	0	0	0	..
			RUBIACEAE						
4384	*Adina cordifolia*	C. G. F.	7/54	l, s	–	0	0	0	..
4383A	*Alibertia edulis*	C. G. F.	7/54	l	–	0	0	0	..
4383B	*Alibertia edulis*	C. G. F.	7/54	s	–	0	0	+	..
4383C	*Alibertia edulis*	C. G. F.	7/54	fr	–	0	0	0	..
4062	*Cephalanthus occidentalis*	McIntosh Co., Ga.	4/54	l	–	0	0	+	+
4004	*Chiococca sp.*	Tingo Maria, Peru	4/54	l, s	–	++	0	0	+
4305	*Diodia teres*	Caroline Co., Va.	6/54	l, s, r	–	0	0	0	..
4366	*Diodia teres*	Richmond Co., Ga.	6/54	l, s, r	+	0	0	0	..
4448	*Diodia Virginiana*	Onslow Co., N. C.	7/54	l, s, r	–	0	0	0	..
4537	*Galium latifolium*	Buncombe Co., N. C.	7/54	l, s, fl, r	–	+	0	0	..
4834	*Galium verum*	Mecklenburg, N. Y.	8/54	l, s, fl, r	–	0	0	0	..
4033	*Gonzalagunia bunchosioides*	Tingo Maria, Peru	4/54	l, s, b	–	0	0	+	++
4012	*Hillia parasitica*	Tingo Maria, Peru	4/54	l, s	–	0	0	0	+

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
		RUBIACEAE							
4635	*Houstonia nigricans*	Bullitt Co., Ky.	7/54	l, s, fl	–	++	0	+++	..
4348	*Pinckneya pubens*	Tift Co., Ga.	6/54	l, s, fr	–	0	+	0	..
4267	*Richardia scabra*	Brunswick Co., N. C.	6/54	l, s, r	–	0	0	0	..
		RUTACEAE							
4714	*Atalantia citroides*	C. G. F.	7/54	l, s, fr	–	0	0	0	..
4152	*Citrus limon*	Chico, Calif.	5/54	l, s	+[a]	+	0	0	+
		SALICACEAE							
4473	*Populus alba*	Brunswick Co., N. C.	7/54	l, s	–	0	0	0	..
4497	*Populus heterophylla*	Bertie Co., N. C.	7/54	l, s	–	0	0	0	..
4841	*Populus tremuloides*	Wayne Co., Pa.	8/54	l, s	–	0	0	+	..
4237	*Salix discolor*	Durham Co., N. C.	6/54	l, s	–	0	0	0	0
4671	*Salix discolor*	Baltimore Co., Md.	7/54	l, s	–	0	0	0	..
		SANTALACEAE							
4136	*Comandra umbellata*	Washington Co., Md.	5/54	l, s	–	0	0	0	0
4541	*Pyrularia pubera*	Henderson Co , N. C.	7/54	l, s, fr	–	0	0	0	..
		SAPINDACEAE							
4005	*Cardiospermum sp.*	Tingo Maria, Peru	4/54	l, s	–	0	0	0	+++
4125	*Sapindus saponaria*	C. G. F.	5/54	l, s	–	0	0	0	++
		SARRACENIACEAE							
4081	*Sarracenia flava*	Bulloch Co., Ga.	4/54	l	–	0	0	0	0
4294	*Sarracenia purpurea*	Anne Arundel Co., Md.	6/54	l, s, r	–	+	0	0	..
		SAPOTACEAE							
4378	*Lucuma ovata*	C. G. F.	7/54	l, s	–	0	0	++	..
		SAURURACEAE							
4064	*Saururus cernuus*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+
		SAXIFRAGACEAE							
4509	*Astilbe biternata*	Haywood Co., N. C.	7/54	l, s, fl, r	–	0	0	+	..
4087	*Decumaria barbara*	Clarke Co., Ga.	4/54	l	–	0	0	0	0
4607	*Heuchera Americana*	Wycoming Co., W. Va.	7/54	l, s, fl, r	–	0	0	+++	..
4534	*Hydrangea arborescens*	Buncombe Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4513	*Hydrangea radiata*	Haywood Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4132	*Saxifraga Virginiensis*	Washington Co., Md.	5/54	l, s, fl, r	–	0	0	0	0
4457	*Itea Virginica*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
		SCROPHULARIACEAE							
4217	*Aureolaria pectinata*	Wake Co., N. C.	6/54	l, s, r	–	0	0	0	0
4310	*Aureolaria Virginica*	Chatham Co., N. C.	6/54	l, s, r	–	0	0	0	..
4460	*Bacopa Caroliniana*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4797	*Cordylanthus filifolius*	Dripping Springs, Calif.	7/54	l	–	0	++	++	..
4779	*Digitalis purpurea*	Baltimore Co., Md.	7/54	l, s, fl, r	–	0	0	0	..
4399	*Gerardia pedicularia*	Atlantic Co., N. J.	7/54	l, s, r	–	0	0	0	..
4391	*Gerardia Virginica*	Hanover Co., Va.	7/54	l, s, fl	+[a]	0	0	0	..

4994	*Macranthera flammea*	Liberty Co., Fla.	8/54	l, s	–	0	0	0	..
4400	*Melampyrum lineare*	Atlantic Co., N. J.	7/54	l, s, fl, r	–	0	0	0	..
4610	*Mimulus ringens*	Mingo Co., W. Va.	7/54	l, s, r	–	0	0	0	..
4098	*Paulownia tomentosa*	Clarke Co., Ga.	4/54	l	–	0	0	0	0
4100	*Paulownia tomentosa*	Clarke Co., Ga.	4/54	l	–	0	0	0	+
4515	*Pedicularis Canadensis*	Haywood Co., N. C.	7/54	l, s	–	0	0	0	..
4260	*Penstemon laevigatus*	Wake Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
4350	*Penstemon multiflorus*	Tift Co., Ga.	6/54	l, s, fl, r	–	0	0	0	..
4956	*Seymeria cassioides*	Leon Co., Fla.	8/54	l, s, r	–	0	0	+	..
4594	*Verbascum blattaria*	Carroll Co., Md.	7/54	l, s, fl, r	+[c]	0	0	0	..
4866	*Veronica anagallis-aquatica*	Harford Co., Md.	8/54	l, s, fl, r	–	0	0	0	..
			SELAGINELLACEAE						
4547	*Selaginella rupestris*	Transylvania Co., N. C.	7/54	l, s	–	+	0	0	..
			SIMAROUBACEAE						
4264	*Ailanthus altissima*	Brunswick Co., N. C.	6/54	l, s	–	0	0	0	..
			SOLANACEAE						
4708	*Cestrum nocturnum*	C. G. F.	8/54	l, s	+[c]	0	0	0	..
4709	*Cestrum parqui*	C. G. F.	8/54	l, s	–	0	+	+++	..
4379A	*Cestrum parqui*	C. G. F.	7/54	l	–	0	0	++	..
4379B	*Cestrum parqui*	C. G. F.	7/54	tw	–	0	0	0	..
4371A	*Cestrum sp.*	C. G. F.	8/54	l, s	+[a]	0	0	0	..
4713B	*Cestrum sp.*	C. G. F.	8/54	fr	+[b]	0	0	0	..
4485	*Datura stramonium*	Nansemond Co., Va.	7/54	l, s	–	0	++	0	..
4641	*Datura stramonium*	Bullitt Co., Ky.	7/54	l, s	–	0	+	0	..
4097	*Lycium halimifolium*	Clarke Co., Ga.	4/54	l	–	0	0	0	+++
4769	*Physalis maritima*	Brunswick Co., N. C.	7/54	l, s, fr, r	–	0	++	0	..
4054	*Physalis viscosa*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+++
4328	*Solanum Carolinense*	Wake Co., N. C.	6/54	l, s, fr, r	–	0	0	0	..
4134	*Solanum dulcamara*	Washington Co., Md.	5/54	l, s	–	0	+	0	0
4738	*Solanum nigrum*	New Hanover Co., N. C.	7/54	l, s, fl, r	+[b]	0	+	0	..
			SPARGANIACEAE						
4583	*Sparganium Americanum*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
			STAPHYLEACEAE						
4674	*Staphylea trifolia*	Harford Co., Md.	7/54	l, s, fl, r	–	0	0	0	..
			STERCULIACEAE						
4123	*Abroma augusta*	C. G. F.	6/54	l, s, fr	–	0	0	0	0
			STYRACACEAE						
4084	*Halesia Carolina*	Clarke Co., Ga.	4/54	l	–	0	0	0	++
4556	*Halesia Carolina*	Macon Co., N. C.	7/54	l, s, fr	–	0	0	0	..
4512	*Styrax grandifolia*	Haywood Co., N. C.	7/54	l, s	–	0	0	0	..
			SYMPLOCACEAE						
4433	*Symplocos tinctoria*	Brunswick Co., N. C.	7/54	l, s	+[a]	+	0	0	..
			TAMARICACEAE						
4469	*Tamarix gallica*	Brunswick Co., N. C.	7/54	l, s	–	+	0	+++	..

Accession No.	Species	Collection: Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			TILIACEAE						
4676	*Tilia Americana*	Harford Co., Md.	7/54	l, s, fr	–	0	0	+	..
4313	*Tilia Floridana*	Chatham Co., N. C.	6/54	l, s, fl	–	0	0	0	..
4024	*Triumfetta sp.*	Tingo Maria, Peru	4/54	l, s, fr	–	0	0	0	+++
			TURNERACEAE						
4953	*Piriqueta Caroliniana*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	0	..
			TYPHACEAE						
4772	*Typha glauca*	Brunswick Co., N. C.	7/54	l, s, fr	–	0	++	++	..
4431	*Typha latifolia*	Brunswick Co., N. C.	7/54	l, s, fr, r	–	0	0	0	..
			ULMACEAE						
4475	*Celtis laevigata*	Elizabeth City Co., Va.	7/54	l, s	–	0	0	0	..
4645	*Celtis occidentalis*	Mason Co., Ky.	7/54	l, s, fl, r	–	0	0	0	..
4621	*Ulmus alata*	Wayne Co., Ky.	7/54	l, s	–	0	0	+	..
4202	*Ulmus Americana*	Wake Co., N. C.	6/54	l, s	–	+	0	0	+
			UMBELLIFERAE						
4555	*Angelica triquinata*	Macon Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4262	*Angelica villosa*	Johnston Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
4236	*Cicuta maculata*	Durham Co., N. C.	6/54	l, s	–	0	0	0	0
4458	*Centella erecta*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4833	*Conioselinum Chinense*	Mecklenburg, N. Y.	8/54	l, s, fl	–	0	+	0	..
4754	*Eryngium aquaticum*	Carteret Co., N. C.	7/54	l, fl, r	–	0	0	0	..
4341	*Eryngium prostratum*	Leon Co., Fla.	6/54	l, s, fl	–	0	0	0	..
4369	*Eryngium yuccifolium*	Emanuel Co., Ga.	6/54	l, s, fl	+[a]	0	0	0	..
4519	*Levisticum officinale*	Buncombe Co., N. C.	7/54	l, s, fl, fr	–	0	+	0	..
4324	*Ligusticum Canadense*	Orange Co., N. C.	6/54	l, s, fl	–	0	0	0	..
4860	*Osmorhiza claytoni*	Frederick Co., Md.	8/54	l, s, r	–	0	0	0	..
4976	*Oxypolis filiformis*	Wakulla Co., Fla.	8/54	l, s, r	–	0	0	0	..
4672	*Petroselinum crispum*	Baltimore Co., Md.	7/54	l, s, r	+[a]	0	0	0	..
4167	*Ptilimnium capillaceum*	Brunswick Co., N. C.	6/54	l, s, fl	–	+	0	0	0
4333	*Sanicula Marilandica*	Wake Co., N. C.	6/54	l, s, r	+[a]	0	+	0	..
4755	*Sium suava*	Carteret Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4642	*Torilis Japonica*	Anderson Co., Ky.	7/54	l, s, r	–	0	0	0	..
			URTICACEAE						
4683	*Boehmeria cylindrica*	Dorchester Co., Md.	7/54	l, s, fl, r	–	0	0	0	..
4270	*Boehmeria drummondii*	Brunswick Co., N. C.	6/54	l, s, r	–	0	0	0	..
4150	*Laportea Canadensis*	Preston Co., W. Va.	5/54	l, s, r	–	0	0	0	++
4673	*Pilea pumila*	Harford Co., Md.	7/54	l, s, r	–	0	0	0	..
4865	*Urtica dioica*	Harford Co., Md.	8/54	l, s, fl	+[a]	0	0	0	..
4814	*Urtica gracilis*	Yates Co., N. Y.	8/54	l, s, r	–	0	0	0	..
			VERBENACEAE						
4068	*Callicarpa Americana*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+
4470	*Lantana camara*	Brunswick Co., N. C.	7/54	l, s, fr	–	0	0	0	..
4058	*Lippia nodiflora*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+
4455	*Verbena bonariensis*	Brunswick Co , N. C.	7/54	l, s, fl	–	0	0	0	..

4085	*Verbena rigida*	Clarke Co., Ga.	4/54	l, s		0		0			0		+	
4619	*Verbena simplex*	Pulaski Co., Ky.	7/54	l, s, fl		0		0			0			
4248	*Verbena urticifolia*	Wake Co., N. C.	6/54	l, s		0		0			0		+	+
		VIOLACEAE												
4045	*Viola papilionacea*	Wake Co., N. C.	4/54	l, s		0		0			0		+	
4135	*Viola pedata*	Washington Co., Md.	5/54	l, s, fl, r										
4143	*Viola striata*	Monongalia Co., W. Va.	5/54	l, s, r	+	0		0			0		+	
		VITACEAE												
4269	*Ampelopsis arborea*	Brunswick Co., N. C.	6/54	l, s		+		0			+			
4022	*Cissus sp.*	Tingo Maria, Peru	4/54	l, s		0		0			0		+	+
4208	*Parthenocissus quinquifolia*	Wake Co., N. C.	6/54	l, s, r		0		0			0			
4442	*Vitis rotundifolia*	Brunswick Co., N. C.	7/54	l, s, fr		0		0			0		+	+
4206	*Vitis vulpina*	Wake Co., N. C.	6/54	l, s		0		0			+			
		XYRIDACEAE												
4573	*Xyris difformis*	Brunswick Co., N. C.	7/54	l, s, fl, r		+		0			0			
		ZINGIBERACEAE												
4711	*Phaeomeria speciosa*	C. G. F.	7/54	l, s, r		0		0			0			
		ZYGOPHYLLACEAE												
4743	*Tribulus terrestris*	New Hanover Co., N. C.	7/54	l, s, fl	[b]	0		0			0			

[a] No steroidal sapogenins found. [b] See text for steroidal sapogenins found.

CORRECTIONS ON ACCESSIONS

CORRECTIONS ON SECOND 1,000 ACCESSIONS (4)

Page	Acces. No.	Should Read
14	1997	*Dioscorea spiculiflora*
14	1999	*Dioscorea floribunda*
27	1709	*Cercocarpus*

CORRECTIONS ON THIRD 1,000 ACCESSIONS (6)

Page	Acces. No.	Should Read
4	2328	*Agave lecheguilla*, var. *Dioscorea Mexicana*
13	2249	

CORRECTIONS ON FOURTH 1 000 ACCESSIONS (7)

Page	Acces. No.	Should Read
655	3341	*Agave deserti*
656	3104	*Agave roezliana*
665	3112	*Dioscorea floribunda*
665	3115	*Dioscorea floribunda*
665	3129	*Dioscorea plumifera*
665	3168	*Dioscorea floribunda*
665	3174	*Dioscorea cf. floribunda*
666	3358	*Dioscorea bulbifera*
666	3359	*Dioscorea bulbifera*
666	3360	*Dioscorea bulbifera*
666	3369	*Dioscorea Mexicana*
668	3547	*Dioscorea floribunda*
668	3551	*Dioscorea floribunda*
669	3736	*Dioscorea convolvulacea*
669	3737	*Dioscorea convolvulacea*
669	3738	*Dioscorea convolvulacea*
669	3739	*Dioscorea densiflora*
669	3742	*Dioscorea n. sp.*
669	3745	*Dioscorea convolvulacea*
669	3746	*Dioscorea convolvulacea*
669	3748	*Dioscorea Mexicana*
669	3749	*Dioscorea convolvulacea*
669	3751	*Dioscorea galeottiana*
669	3755	*Dioscorea lobata* var. *morelosana*
670	3758	*Dioscorea convolvulacea*
670	3849	*Acalypha sp.*
670	3950	*Acalypha sp.*
670	3057	*Alchornea*
673	3586A	*Yucca filamentosa*
678	3276	*In Phytolaccaceae*
678	3681	*Polystichum acrostichoides*
679	3118	*Poterium sanguisorba*

Flavonoids were found in 96 species, of which at least half seem to be new to the record.

Alkaloids were found in 86 species, an incidence of about 9% of the total. This is higher than noted in previous series, due largely to the change in technique mentioned above. Most of the species were wild plants growing in the eastern United States. Some 45 species are new to the record.

Tannins were again conspicuously absent from 150 species of Compositae. There were no noteworthy occurrences of tannin except in *Rhus*, and here it is expected.

Unsaturated sterols have proved to be so ubiquitous that the test for them was made on only a portion of the accessions.

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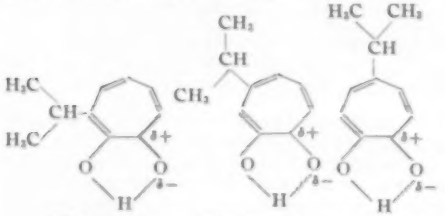
A Pharmacological Study of Gamma-Thujaplicin*

By JOHN E. HALLIDAY

Gamma-thujaplicin (2-hydroxy-5-isopropyl-2,4,6-cycloheptatrien-1-one), a tropolone derivative occurring in western red cedar, has been studied pharmacologically in the form of its sodium salt (T-Na). It has been found to exert a combination of stimulant and depressant actions on the mammalian central nervous system and only depression in frogs. Stimulant effects, in the form of convulsions shown to be of cerebral origin, are evident only in untreated animals. Certain signs of depression can also be observed in untreated animals while further depressant actions are revealed only in animals which have been treated with narcotic drugs. T-Na reduces the responses of isolated frog skeletal muscle and antagonizes the action of some stimulant drugs on isolated intestinal muscle.

GAMMA-THUJAPLICIN (2-hydroxy-5-isopropyl-2,4,6-cycloheptatrien-1-one) is one of three isomeric isopropyltropolones which occur in the heartwood of western red cedar (*Thuja plicata* D. Don.). The term tropolone is used to designate the seven-membered carbon ring compound 2-hydroxy-2,4,6-cycloheptatrien-1-one, derivatives of which are relatively rare in nature. The thujaplicins, which were the first naturally-occurring compounds to be characterized conclusively as monocyclic tropolones (1) and are the simplest of the natural tropolone derivatives, have the following structures:



α-Thujaplicin β-Thujaplicin γ-Thujaplicin

Other substances found in nature containing the tropolone structure include the mold metabolites stipitatic and puberulic acids, and the alkaloid colchicine (2).

The chemistry of the thujaplicins and other tropolone derivatives has been studied quite extensively (2, 3). Antimicrobial studies of the thujaplicins have shown that all of the isomers possess antifungal and antibacterial properties and it is interesting to note that their antifungal activity has been given as the reason for the durability of cedar wood (4, 5, 6). The only pharmacological studies of thujaplicins which have been reported appear to be those by Lee (7, 8, 9) and other Japanese workers (10, 11, 12) on β-thujaplicin. This isomer is commonly called hinokitiol by the Japanese, who obtain it from the tree known as Formosan hinoki (*Chamaecyparis taiwensis*, Masamune et Suzuki—*Chamaecyparis obtusa*, Sieb. et Zucco. F. *Formosana*, Hayata). Lee found that its actions were largely depressant in nature though low concentrations may stimulate some organs, e.g., isolated heart and rabbit uterus. It was suggested by Katsura (13), who has summarized Lee's work, that β-thujaplicin is a nerve-muscle poison related in its actions to camphor and thujone, and other terpene compounds. Since there is a lack of pharmacological information on γ-thujaplicin, and since supplies of this isomer were available, the present investigation was undertaken.

Gamma-thujaplicin was obtained as a white crystalline substance melting between 79 and 80°. As the free substance is only slightly soluble in water, the sodium salt was used in the experiments described here. This was precipitated from a concentrated solution of γ-thujaplicin in alcohol by the addition of a freshly prepared alcoholic solution of sodium ethylate.

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The author is indebted to Dr. J. G. Gardner of the Vancouver Laboratory, Forest Products Laboratories of Canada, for supplies of γ-thujaplicin used in this study, and to Mr. J. G. Moir for assistance in preparing photographs.
Appreciation is expressed to Abbott Laboratories Ltd., Ciba Company Ltd., Montreal, and to E. R. Squibb & Sons of Canada Ltd., for supplying some of the drugs used in this investigation.

The precipitate was collected and dried, then powdered and washed with successive portions of anhydrous ether, and again dried, to give a yellow powder. γ-Thujaplicin sodium (T-Na) dissolves readily in water to form alkaline solutions, the pH of a 1% solution being 8.4.

EXPERIMENTAL PROCEDURES AND RESULTS

Effects in Intact Animals.—Most of the observations of the effects of T-Na in intact, conscious animals were made following injection of a 1 or 5% aqueous solution into Swiss albino mice, Sprague-Dawley rats, and rabbits. Six cats and two dogs were also injected.

In all five species of mammals T-Na produced convulsions of a mixed clonic and tonic type. Doses producing convulsions in the various species, expressed as mg. per Kg. of body weight, were: mice and rats, i. p., 75 to 100, oral, 150 or more; rabbits i. v., 25 to 40; cats i. p., 50 to 70; dogs i. v., 30 and 40. Depending on the dose, one to several convulsive seizures occurred, lasting from five to fifteen seconds and recurring at intervals which varied from fifteen to ninety seconds. The convulsions were preceded by a brief period of ataxia and followed by symptoms of depression which included slowing of reflexes, decrease in muscle tone and in voluntary movement, and absence of certain placing reactions, such as the dorsal contact reaction, the chin reaction, and the edge reflex. During this period the animals would lie either prone or on one side, with limbs extended and somewhat flaccid. No attempts to move or regain a normal position were made. When the larger animals were placed on their feet they collapsed again to a lying position. Righting reflexes were reduced but not abolished. In rodents, a decrease in respiratory rate, usually accompanied by an increase in respiratory depth, was also observed. In animals in which these effects were marked, the appearance was one of slow, labored breathing. The duration of the postconvulsive depression varied with the dose, most signs of depression disappearing within twenty minutes following the last seizure. In rodents, respiration was the last function to return to normal, often taking one to three hours. When death occurred from the larger doses, it was always during the post-convulsive depressive stage following a period of gradually declining respiratory activity, and never during the convulsive seizure as is the case with such convulsive agents as metrazol.

T-Na was also administered to frogs, either by way of the dorsal lymph sac or via the abdominal vein. Here the effect of T-Na was one of depression only. Doses of 50 to 500 mg./Kg. caused the frogs to slump to a prone position with reduced reflex activity and respiratory movements, but no initial stimulant effect was observed. Death occurred in all frogs which received more than 100 mg./Kg.

Acute Toxicity and Convulsive Dose in Mice.—The intraperitoneal LD_{50} and CD_{50} (50% convulsive dose) were determined in Swiss albino mice which had been fasted for twelve hours. For each determination seven groups of 10 mice each were used. All deaths were recorded which occurred during a period of four days following administration of T-Na. The mice in which death was delayed were in a state of depression, following convulsions, which deepened progressively, terminating in respiratory paralysis. There were no deaths after the fourth day. When calculated according to the graphic method of Miller and Tainter (14) the intraperitoneal LD_{50} was found to be 162 ± 3.2 mg./Kg. and the intraperitoneal CD_{50} 73.5 ± 1.8 mg./Kg.

Site of Convulsive Action.—Six Sprague-Dawley rats, after ligation of the common carotids under ether anesthesia were decerebrated according to methods described in the literature (15, 16). Five other rats, with the common carotids ligated but otherwise intact, served as controls. After allowing at least two hours to elapse for elimination of ether, T-Na was administered intraperitoneally. Doses from 150 to 300 mg./Kg. failed to elicit convulsions in any of the decerebrate rats though convulsions were produced in all of the control rats, including two rats in which the dose was only 100 mg./Kg. Decerebrate rigidity, which was present to a marked degree in three of the decerebrate rats and to a lesser degree in the others, was not significantly altered by T-Na. These results, which indicate a cerebral site for the convulsant action of T-Na, were supported by observations in four decerebrate cats used in later experiments for reflex studies. No convulsive activity was produced in any of these cats by intravenous doses of from 40 to 75 mg./Kg.

Interaction of T-Na with Hypnotic Agents.—The possibility that the convulsive action of T-Na would antagonize the action of hypnotic drugs was investigated by determining its effect on the sleeping times of mice following administration of pentobarbital sodium, thiopental sodium, and chloral hydrate. For comparison with a known analeptic agent, the effect of metrazol on the sleeping time was also determined. Ten mice were used at each dosage level. T-Na was injected intraperitoneally immediately following administration of the barbiturates and ten to fifteen minutes following administration of chloral hydrate. Sleeping time was taken as the period from the time the mice first could be placed on their backs until they could turn from their backs to an upright position. The results, shown in Table I, indicate that T-Na does not possess analeptic properties. In fact, an apparent prolongation of sleeping times occurred in some groups when T-Na was administered.

Action on Spinal Reflexes.—The possibility that muscle hypotonia seen in animals which had been given T-Na was due to depression of interneuronal transmission of impulses was investigated by determining the effect of T-Na on the flexion reflex in the cat. Six experiments were performed on cats lightly anesthetized with 0.75 cc. to 0.80 cc. of Dial[1] with urethane per Kg. Four experiments were performed on decerebrate cats and six on spinal cats, prepared under ether anesthesia according to methods described by Burn (17). Artificial respiration was maintained by means of a Palmer variable stroke Ideal respirator pump. The method of recording the flexion reflex was a modification of those described by Slater, *et al.* (18), and Kaada (19) with reflex contractions of the anterior tibealis

[1] Trademark of Ciba Co. Ltd.

TABLE I.—EFFECT OF T-Na ON DURATION OF SLEEPING TIMES PRODUCED BY CENTRAL DEPRESSANTS IN MICE

Depressant Dose and Route	T-Na, mg./Kg., i. p.	Metrazol, mg./Kg., i. p.	No. of Mice	Sleeping Time, min. ± S. D.
Thiopental Na,	..	..	10	14.5 ± 11.5
50 mg./Kg., i.p.	50	..	10	21.2 ± 14.8
	100	..	10	54.3 ± 22.7
	200	..	6[a]	245.8 ± 22.9
	..	50	10	3.8 ± 3.5[b]
Pentobarbital Na	..	..	10	113.3 ± 47.6
75 mg./Kg., i.v.	50	..	10	122.6 ± 53.1
	100	..	10	106.3 ± 26.8
	200	..	10	208.5 ± 79.6
	..	100	10	36.6 ± 21.0
Chloral Hydrate	..	..	10	58.0 ± 22.8
400 mg./Kg., i.p.	50	..	10	101.3 ± 40.5
	100	..	10	221.9 ± 70.0
	200	..	5[c]	261.4 ± 56.7
	..	100	10	10.3 ± 6.2

[a] Number of survivors out of 10 mice injected.
[b] Mean sleeping time for 5 mice; the remaining 5 mice of this group did not lose the righting reflex.
[c] Number of survivors out of 10 mice injected.

muscle being elicited by stimuli applied every twenty-five seconds through a shielded electrode secured to the central end of the cut tibeal nerve. In six experiments responses of the opposite tibealis anterior to stimuli applied at twenty-five-second intervals to the peripheral end of the cut sciatic nerve were recorded concurrently with the flexion reflex. The stimulus used for eliciting the flexion reflex consisted of a tetanic induction shock of approximately 0.5 seconds duration from an inductorium; single break shocks were used for sciatic nerve stimulation. In the decerebrate and spinal cats at least two hours were allowed to elapse between removal of ether and beginning the record. The effect of T-Na was compared to that of mephenesin, a drug known to inhibit internuncial transmission. Both drugs were injected intravenously.

In the anesthetized cats, T-Na in doses of 30 and 40 mg./Kg. inhibited the flexion reflex to a degree comparable to that produced by similar doses of mephenesin (Fig. 1). In the decerebrate and spinal cats however, T-Na had an insignificant depressant action compared with that produced by mephenesin (Figs. 2 and 3). T-Na had no effect on the responses of the contralateral muscle to stimulation through its motor nerve.

Action on Frog Peripheral Nerve and Skeletal Muscle.—The nerve muscle preparation used here consisted of the iliofibularis muscle with its attached motor nerve and parent sciatic trunk, of which the method of isolation and use have been described by

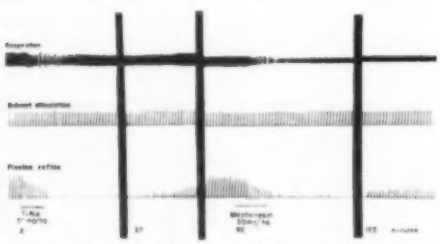

Fig. 1.—Anesthetized cat. Action of T-Na and mephenesin on tibealis anterior responses.

Fig. 2.—Decerebrate cat. Action of T-Na and mephenesin (*M*) on flexion reflex.

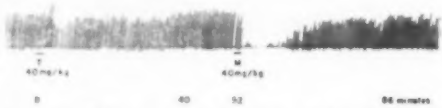

Fig. 3.—Spinal cat. Action of T-Na (*T*) and mephenesin (*M*) on flexion reflex.

Quilliam (20). Contractions were recorded with the preparation immersed in oxygenated frog Ringer's solution contained in a Palmer horizontal muscle chamber. When nerve and muscle were to be isolated, one from the other, the bath was divided by a specially constructed plexiglass partition. Stimuli consisted of single break shocks from an inductorium and were applied either directly to the muscle, to the motor nerve, or alternately to nerve and muscle at thirty-second intervals.

When T-Na was mixed into the bathing solution a gradual decline in the height of the muscle contractions occurred. If T-Na was left in the bath long enough the responses would decline eventually to zero or close to it. Recovery was slow, even after repeated washings, and seldom did the contractions regain the original height. Depression of responses occurred whether the muscle was stimulated directly or via its motor nerve, though responses to nerve stimulation were affected to a greater degree. When the nerve and muscle were in separate baths it was shown that the greater susceptibility of indirectly elicited responses was not due to depression of conduction in the motor nerve (Fig. 4).

Action on Blood Pressure and Heart Rate.—Blood pressure was recorded from the carotid artery in four cats and three dogs, anesthetized with pentobarbital sodium. Heart rate was determined

Fig. 4.—Isolated frog sciatic nerve-ilio-fibularis muscle preparation, nerve and muscle in separate chambers. Stimulus applied alternately to nerve or to muscle directly. This record shows the effect of T-Na 20 mg. % (*T*20) and of T-Na 10 mg. % (*T*10) when added to nerve chamber (*n*) or to muscle chamber (*m*). The *m* above the record indicates that the higher responses are those due to direct stimulation of the muscle.

from cardiograms recorded from lead II on an Edin electronic cardiograph, model 210. Intravenous injection of T-Na in doses of 25 to 50 mg./Kg. produced a temporary fall in blood pressure accompanied by a decrease in heart rate. The extent and rapidity of the fall in blood pressure, for a given dose, varied with the speed of injection. Both effects were more pronounced in cats than in dogs. Large doses (50 mg./Kg.), whether injected rapidly or slowly, caused complete paralysis of respiration. This effect was reversible and respiratory function returned after periods of fifteen to twenty minutes on artificial respiration (Fig. 5). The hypotensive effect remained unchanged after atropinization or bilateral section of the vagus. Responses to carotid occlusion and to injected epinephrine were not altered by an immediately previous injection of T-Na.

Action on Isolated Intestine.—Segments of ileum from rabbits and guinea pigs were suspended in a 50-cc. muscle chamber containing oxygenated Tyrode's solution and recordings made of their longitudinal contractions. The effect of T-Na was determined on spontaneous contractions and on the responses to the following intestinal stimulants: acetylcholine chloride, histamine acid phosphate, nicotine sulfate, barium chloride, and 5-hydroxytryptamine creatinine sulfate (serotonin). The final concentrations of the drugs in the muscle bath are expressed as mg. % of these salts.

In concentrations of 5 and 10 mg. % T-Na depressed spontaneous contractions of both rabbit and guinea-pig ileum with little or no effect on tone. On rabbit intestine, depression of spontaneous movements was much more pronounced when the contractions were of high magnitude. The responses to all of the stimulant drugs were inhibited, but not to the same extent, the responses to nicotine being inhibited most easily. On rabbit ileum this difference was such that T-Na appeared to be almost a selective antagonist of nicotine (Fig. 6). On guinea-pig ileum (Fig. 7) and when higher concentrations were used on rabbit ileum, a selective action against nicotine was less obvious.

DISCUSSION AND SUMMARY

The experimental evidence obtained in this investigation indicates that γ-thujaplicin sodium exerts a mixture of stimulant and depressant actions on the central nervous system of mammals. The stimulant component of its action is evident only in conscious animals and is manifested

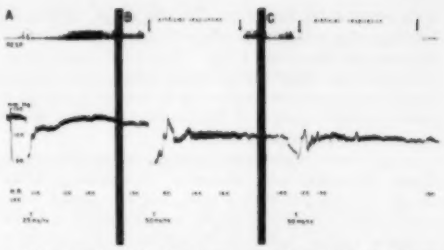

Fig. 5.—Anesthetized cat. Action of T-Na (*T*) on respiration, blood pressure, and heart rate (*H. R.*). In B and C artificial respiration was maintained until spontaneous respiratory movements were evident. Twenty minutes elapsed between A and B, sixteen minutes between B and C.

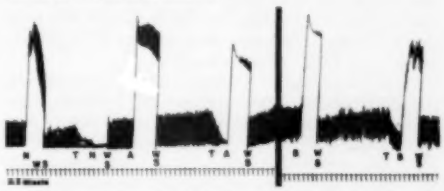

Fig. 6.—Isolated rabbit ileum. Effect of 10 mg. % T-Na (*T*) on responses to 150 mcg. % nicotine sulfate (*N*), 4 mcg. % acetylcholine (*A*), and 25 mg. % barium chloride (*B*). *W* is wash. Drum stopped at *S*.

Fig. 7.—Guinea ileum. Effect of 5 mg. % T-Na (*T*) on responses to nicotine 500 mcg. % (*N*), acetylcholine 4 mcg. % (*A*), and serotonin 100 mcg. % (*S*). *W* is wash.

by convulsions which have been shown to be of cerebral origin. In this respect T-Na is similar to other volatile, cyclic ketones from natural sources, such as camphor and thujone. The depressant component of the action of T-Na appears to affect various levels of the mammalian central nervous system. The abolition of placing reactions observed in rats, rabbits, and cats indicates that cortical areas are depressed since a strictly localized cortical control of these reactions has been demonstrated in the cat by Bard (21) and in the rat by Brooks (22). In rodents, there was a suggestion that respiratory centers might be depressed by T-Na. Other depressant effects, which could be observed only when T-Na was given to previously narcotized animals, included the respiratory paralysis produced in pentobarbitalized cats and dogs, and the interneuronal blockade seen in anesthetized cats but not in spinal and decerebrate cats.

The results of the experiments in which T-Na was administered in conjunction with depressant drugs to mice show that T-Na is of no value as an analeptic. Toxic effects are involved in the alteration of sleeping times by the 200 mg./Kg. dose of T-Na. The prolongation of thiopental and chloral

hydrate sleeping times by T-Na in doses of 50 and 100 mg./Kg. may be further evidence of a central depressant action for T-Na. However, since lower doses did not alter the sleeping times of all of the depressants used, it cannot be generally concluded that nontoxic doses of T-Na prolong sleeping times produced by depressant drugs.

It has been reported that the central stimulant drugs picrotoxin and metrazol also possess some central depressant action (23), but compared with these useful analeptics, T-Na would appear to have a much higher ratio of depressant to stimulant activity.

In frogs, the depressant action of T-Na is predominant and no signs of stimulation could be seen when the drug was injected intravenously or intralymphatically. There may be a similarity here to the action of camphor, which produces a pronounced paralytic action on the spinal cord of frogs which entirely obscures other actions it may have higher up in the nervous system (24).

On other organs and systems which were studied, T-Na exhibited only depressant effects. The temporary hypotension seen in anesthetized cats and dogs, and which was intensified when the drug was rapidly injected, seems most likely to be due to a direct depression of the myocardium resulting from its exposure to high concentrations of T-Na. A central vagal action is ruled out by the persistence of the hypotensive response after atropinization and bilateral section of the vagi. Interruption of sympathetic pathways does not seem likely since the responses to carotid occlusion and exogenous epinephrine were not altered by T-Na. On the other hand, bradycardia following injection of T-Na paralleled the hypotensive response.

Although there was no evidence that T-Na inhibited neuromuscular conduction in the cat anterior tibealis *in situ*, there was a suggestion of such an action on the isolated frog nerve-muscle preparation. On this preparation, contractions due to stimulation of the motor nerve were reduced to a greater degree than those due to direct stimulation of the muscle. This, together with the fact that T-Na did not inhibit conduction in the motor nerve, would appear to indicate a depressant effect at the neuromuscular junction. This action, however, is not a potent one and develops slowly. It may be that another point of similarity in the actions of T-Na and camphor is indicated here, since camphor has been reported to produce neuromuscular paralysis in frogs but not in mammals (25).

The depressant effect of T-Na on intestinal muscle obviously is not due to an atropine-like mechanism since atropine antagonizes exogenous acetylcholine more readily than it does nicotine, and furthermore, it does not prevent the stimulating effect of nicotine on rabbit intestine (26, 27, 28). On both guinea-pig and rabbit intestine, T-Na inhibited responses to nicotine more easily than those to other intestinal stimulants. Since nicotine is the only one of the stimulant agents which, when applied exogenously, acts primarily to stimulate ganglia, it appears that T-Na, as well as depressing intestinal muscle, exerts also a depressant effect on myenteric ganglia. Naess and Schanche attributed a similar type of action on guinea-pig intestine to barbiturates, which they found to inhibit nicotine responses more than those of other stimulant drugs (29). In view of the relatively high concentrations of T-Na required and the dissimilarity of structure between it and potent ganglionic-blocking agents such as hexamethonium, T-Na does not likely act as a competitive inhibitor at the ganglia. Instead, this action is looked upon as another example of the general depressive action which has been exhibited on other tissues by T-Na.

Although no attempt was made in this investigation of γ-thujaplicin to duplicate the experiments by Lee (7, 8, 9) on β-thujaplicin, a limited comparison of central actions may be made. Lee's reports indicate that on the central nervous system of mice, guinea pigs, and rabbits, β-thujaplicin, like T-Na, produces a combination of stimulant and depressant effects. In the case of the beta isomer however, the depressant component of the central action appears to be more predominant. For example, Lee's results show that subcutaneous doses of 500 mg./Kg. of β-thujaplicin often caused death in guinea pigs without previous convulsions.

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Veratrum Alkaloids XXXV*

The Hypotensive Principles of Cryptenamine, A *Veratrum viriae* Alkaloid Preparation

By S. MORRIS KUPCHAN and NORBERT GRUENFELD†

Cryptenamine has been reported to have a more favorable ratio of emetic to hypotensive dose than other veratrum preparations, and the advantageous ratio has been attributed to the presence of new hypotensive alkaloids. Cryptenamine was fractionated with the aid of partition column chromatography, adsorption column chromatography, and paper partition chromatography. The known hypotensive alkaloids protoveratrine A, protoveratrine B, germitrine, neogermitrine, germerine, and germidine were isolated. In addition, the relatively nonhypotensive alkaloids jervine, rubijervine, isorubijervine, and an apparently new noncarbonyl alkamine were obtained. Paper chromatographic evidence suggesting the presence of the hypotensive alkaloids germbudine and neogermbudine and the relatively nonhypotensive veratramine was also obtained.

PURE veratrum alkaloids and their preparations have been administered in recent years for the treatment of hypertension. The deterrent to the wider use of these agents in therapy is the narrow range between their therapeutic and emetic doses (1).

Cryptenamine, an alkaloidal preparation obtained from *Veratrum viride* by a nonaqueous benzene-triethylamine extraction procedure has been reported to have a ratio of emetic to effective hypotensive dose superior to that of other veratrum preparations (2–5). In humans, the divergence between the hypotensive and the emetic doses was most apparent on intravenous administration (5). The satisfactory ambulatory treatment of arterial hypertension by oral administration of cryptenamine has also been reported (6). However, Abreu, *et al.* (7), reported in 1954 that cryptenamine did not demonstrate a ratio of emetic to hypotensive dose superior to that of protoveratrine A when tested in dogs.

The advantageous pharmacological properties of cryptenamine have been attributed to the presence of new amorphous hypotensive ester alkaloids reportedly lost by hydrolysis during the alternative aqueous ammonia-benzene extraction procedure (2–4). It was the purpose of this investigation to identify the components of cryptenamine which are responsible for its hypotensive activity.

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Ultraviolet absorption spectra were determined in 95% ethanol on a Cary recording spectrophotometer (model 11MS). Infrared spectra were determined in chloroform solution (unless otherwise specified) on a Baird double beam infrared recording spectrophotometer. Paper chromatography was conducted by the descending technique on Whatman No. 1 paper. Microanalyses were carried out by Dr. S. M. Nagy and his associates at the Massachusetts Institute of Technology and Huffman Microanalytical Laboratories, Wheatbridge, Colorado, on samples dried under reduced pressure.

Paper Chromatography.—The procedure and solvent systems employed were essentially those described by Fischbach and Levine (8, 9) for an ascending technique. This method involves the use of prewetted buffered paper and the detection of alkaloidal spots with a chloroform solution of bromphenol blue. Paper was impregnated with pH 3.5 McIlvaine buffer solution and dried. Before use this was rewetted with acetone-water (3:2) and samples (*ca.* 2 μl. of 3% solutions in chloroform) were applied. Approximately fifteen minutes after rewetting, the paper was placed into a tank equilibrated with respect to developing solvent and water. After a development period of four to five hours the solvent front had traveled about 15 inches. The paper was dried, sprayed with indicator, and exposed to a flow of steam. Cryptenamine alkaloids gave blue, blue-green, or purple spots on a yellow background; the color given by each alkaloid helped in its identification by comparison with the behavior of known compounds.

The three solvent systems (8, 9) employed in this investigation were: (*A*) *n*-butanol, *n*-butyl acetate, formic acid (5:25:1); (*B*) ethylene chloride, cellosolve acetate, pyridine (15:10:1); and (*C*) isooctane, methyl isobutyl ketone, pyridine (20:10:2). System *A* was employed to follow the course of most partition and adsorption chromatography column separations. Systems *A*, *B*, and *C* were used for comparison of cryptenamine components with authentic samples of known alkaloids.

Fractionation of Cryptenamine by Partition Chromatography.—Partition columns employing Celite 545 as support for the stationary phase consisting of a pH 4.25, 0.5 *M* citrate buffer were used for the frac-

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tionation of cryptenamine. A slurry of Celite 545 (120 Gm.) was made with benzene (1,000 cc.) and the buffer solution (100 cc.) was added in portions; the mixture was stirred vigorously after each addition. The hydrated Celite was then packed into a column (3 cm. diameter) in the usual manner and a solution of cryptenamine[1] (2 Gm., batch CaC64, in 3 cc. of chloroform) was added to the column. The column was eluted with appropriate solvents at a rate of *ca.* 2 cc. per minute and 100 cc. fractions were collected. The solvent was evaporated and the contents of each flask were weighed and submitted to paper chromatographic analysis using solvent system *A*. The contents of fractions demonstrating a similar composition were combined and aliquots were submitted for pharmacological testing to ascertain hypotensive activities.[2]

Variations were observed in the course of duplicate separations, probably due to differences in the degree of packing of columns, and thus the results obtained in two fractionations of the components of cryptenamine are summarized separately in Tables I and II. The pharmacological data listed for the first column separation (Table I) indicates that the hypotensive principles of cryptenamine were concentrated in a few fractions. The pharmacological data for the second separation demonstrated a similar distribution of hypotensive activity.

Identification of Isolated Components.—Components were identified in all cases by comparison of infrared spectra and oi paper chromatographic behavior in three solvent systems with those of authentic samples of known veratrum alkaloids. In addition, the melting points were not depressed on admixture with authentic specimens of the characterized alkaloids. Any additional confirmatory evidence is given individually for each component.

Component I: Isorubijervine.[3]—Chloroform and acetone were added to fraction 1-A (Table I). Upon standing, needles [275 mg., m. p. 237–242° (decompn.)] separated; $[\alpha]_D^{25}$ +8° (c 1.02, ethanol). This compound was identified as isorubijervine, and was also isolated (218 mg.) from fraction 2-B (Table II) by the same crystallization procedure.

Component II: The New Alkamine.—Addition of acetone to fraction 1-B (Table I) led to the separation of white velvety needles (85 mg.). Recrystallization from chloroform-methanol gave needles (45 mg.), m. p. 258–260° (decompn.), $[\alpha]_D^{25}$ +9° (c 1.1, pyr.). This compound demonstrated only end absorption in the ultraviolet and no absorption in the carbonyl region of the infrared spectrum.

Anal.—Calcd. for $C_{27}H_{43}NO_3$: C, 75.48, H, 10.09; N, 3.26; equiv. wt., 429.6. Found: C, 75.39, 75.83; H, 10.02, 10.38; N, 3.14; equiv. wt., 434.

The equivalent weight was determined by a nonaqueous titration method similar to that of Parks and Mitchner (11). Approximately 10 mg. of alkaloid was dissolved in glacial acetic acid (10 cc.) and 2 cc. aliquots were titrated with standard 0.01 *N* $HClO_4$ in glacial acetic acid using *p*-naphtholbenzein[4] (0.1% in glacial acetic acid) as indicator. The solution was titrated to the appearance of a green color, the indicator changing from pink to green.

This apparently new alkaloid, also isolated from fraction 2-E (Table II), demonstrated no hypotensive activity on the dog at an i. v. single dose of 4 mcg./Kg.

Component III: Neogermitrine.—Fraction 1-C (Table I) in benzene was chromatographed on Merck acid-washed alumina (4 Gm., 0.7-cm. diameter column). Elution with mixtures of benzene and chloroform (from benzene to chloroform-benzene, 3:1) yielded a product (59 mg.) shown to be predominantly component III on paper chromatographic analysis. Crystallization from ether gave a compound [prisms, 20 mg., m. p. 235–237° (decompn.)] identified as neogermitrine. Neogermitrine [18 mg., m. p. 239–241° (decompn.)] was obtained in a similar fashion by purification of fraction 2-H (Table II).

Component IV: Protoveratrine A.—Benzene solutions of fraction 2-F (50 mg.) and fraction 2-E (47 mg. remaining after removal of component II by crystallization) were combined and chromatographed on Merck acid-washed alumina (2.5 Gm., 0.7-cm. diameter column). Elution with chloroform-benzene (1:1, 250 cc.) yielded a product consisting mainly of component III (neogermitrine). Further elution with benzene-chloroform (from 65% chloroform to 100% chloroform) yielded a crude product (23 mg.) consisting mainly of component IV. Crystallization from ether led to isolation of protoveratrine A [prisms, 8 mg., m. p. 269–270° (decompn.)].

Component V: Jervine.[3]—Cryptenamine (2 Gm. in benzene) was chromatographed on acid-washed alumina (50 Gm.). The column was eluted with benzene-absolute methanol (99:1, 700 cc.), chloroform (300 cc.), chloroform-absolute methanol (99:1, 600 cc.), chloroform-absolute methanol (98:2, 350 cc.), chloroform-absolute methanol (95:5, 600 cc.), and chloroform-absolute methanol (90:10, 300 cc.). The eluates of the last two solvent combinations were combined on the basis of their paper chromatographic behaviors and evaporated to dryness. Addition of acetone to the residue led to the crystallization of a compound [needles, 33 mg., m. p. 245–246° (decompn.)], $[\alpha]_D^{25}$ −165° (c 1.17, ethanol), identified as jervine.

Component VI: Germitrine.—The continued elution of the alumina column described for the isolation of component III (from fraction 1-C) with benzene-chloroform (75% chloroform to pure chloroform) led to the isolation of a product (60 mg.) consisting predominantly of component VI. Crystallization from ether-petroleum ether yielded germitrine [prisms, 10 mg., m. p. 220–222° (decompn.)].

Germitrine was also isolated from fraction 2-J (Table II) by chromatography on acid-washed alumina (3.5 Gm.). Elution with benzene-chloroform (3:1) removed colored nonalkaloidal material. Further elution with benzene-chloroform (1:1) yielded mainly component VI (32 mg.). Crystallization from ether and recrystallization from same solvent gave germitrine [15 mg., m. p. 227–229° (decompn.)].

[1] We wish to thank Dr. C. J. Cavallito of Irwin, Neisler and Co. for supplying us with the cryptenamine used for this investigation.

[2] We wish to express our gratitude to Dr. Thomas B. O'Dell of Irwin, Neisler and Co. for the pharmacological data reported in this paper. Assays were performed by a procedure previously described (2).

[3] Dr. Allan P. Gray of Irwin, Neisler and Co. has kindly informed us of his unpublished independent isolation of isorubijervine, jervine, rubijervine, and veratramine from cryptenamine.

[4] We wish to thank Dr. Kenneth A. Connors for suggesting the use of this indicator.

TABLE I.—FRACTIONATION OF CRYPTENAMINE[a] BY PARTITION CHROMATOGRAPHY
Experiment 1

Fraction	Eluent	Weight, mg.	Hypotensive Units/mg.[b]	Characterized Components: Alkaloids[c]	Characterized Components: Estimated % of Total[d]
1-A	Benzene, 500 cc.	450	Relatively inactive[e]	I	90
1-B[f]	Benzene, 400 cc.	215	200	II, III, IV	90
1-C[f]	Benzene, 1,100 cc.	220	900	III, V, VI, VII	90
1-D	Benzene, 800 cc.	135	650	V, VI, VII, VIII	100
1-E	Benzene, 400 cc.	75	Relatively inactive	V, VII, VIII	100
1-F	Benzene, 800 cc.	165	Relatively inactive	V, VII, VIII	90
1-G	Benzene, 700 cc.	90	Relatively inactive	V, VII, VIII	70
1-H	Benzene, 1,500 cc.	130	250	IX	40
1-I	Benzene, 5,300 cc.	175	200	IX, X	90
1-J	Chloroform, 800 cc.	230	250	IX, X, XI	70
1-K	Chloroform-methanol, 98:2, 2,000 cc.	140	Relatively inactive	XII, XIII	25
Total		2,025			80

[a] Batch CaC64, Irwin, Neisler and Co., (2 Gm.), C.S.R. unit = 4.0 mcg./Kg.
[b] Number of hypotensive units/mg. = 1,000/C.S.R. unit (mcg./Kg.). One C.S.R. unit represents the amount of intravenously administered hypotensive agent per Kg. of body weight which just abolishes the pressor response to the carotid sinus reflex in dogs (10).
[c] I, isorubijervine; II, new alkamine; III, neogermitrine; IV, protoveratrine A; V, jervine; VI, germitrine; VII, veratramine; VIII, rubijervine; IX, germidine; X, germerine; XI, protoveratrine B; XII, germbudine; XIII, neogermbudine.
[d] The per cent of each fraction that the characterized components represent was estimated from the paper chromatogram of the fraction.
[e] No blood pressure fall observed with i. v. single dose of 4 mcg./Kg.
[f] Paper chromatographic analysis with solvent system *C* indicated the presence of a component not apparent with solvent system *A*. The presence of component IV was detected with solvent system *B*.

TABLE II.—FRACTIONATION OF CRYPTENAMINE[a] BY PARTITION CHROMATOGRAPHY
Experiment 2

Fraction	Eluent	Weight, mg.	Characterized Components: Alkaloids[b]	Characterized Components: Estimated % of Total[c]
2-A	Benzene, 200 cc.	96	I	50
2-B	Benzene, 200 cc.	289	I	100
2-C	Benzene, 200 cc.	70	I	75
2-D	Benzene, 200 cc.	30	I, II	75
2-E	Benzene, 400 cc.	171	II, III	85
2-F[d]	Benzene, 200 cc.	50	II, III, IV	90
2-G[d]	Benzene, 200 cc.	28	III, IV, V, VII	90
2-H	Benzene, 500 cc.	94	III, V, VII	100
2-I	Benzene, 400 cc.	59	III, V, VI, VII	100
2-J	Benzene, 800 cc.	148	V, VI, VII	100
2-K	Benzene, 500 cc.	91	V, VI, VII, VIII	100
2-L	Benzene, 1,100 cc.	178	V, VII, VIII	100
2-M	Benzene, 700 cc.	111	V, VII, VIII	90
2-N	Benzene, 400 cc.	40	V	50
2-O	Benzene, 1,600 cc.	61	None	0
2-P	Benzene, 1,400 cc.	61	IX	40
2-Q	Benzene, 600 cc.	31	IX	70
2-R	Benzene 600 cc.	21	IX	100
2-S	Benzene, 4,350 cc.	37	IX, X	50
2-T	Benzene-chloroform, 3:1, 1,500 cc.	76	X	80
2-U	Benzene-chloroform, 1:1, 1,500 cc.	79	None	0
2-V	Benzene-chloroform, 1:1, 1,500 cc.	51	XI	80
2-W	Benzene-chloroform, 1:3, 1,000 cc.	31	XII, XIII	20
2-X	Chloroform, 1,500 cc.	57	XII, XIII	30
2-Y	Chloroform-methanol, 98:2, 2,000 cc.	55	XII, XIII	25
Total		2,015		77

[a] See footnote [a], Table I. [b] See footnote [c], Table I. [c] See footnote [d], Table I. [d] See footnote [f], Table I. In this fractionation, components IV and VI were clearly separated.

Component VIII: Rubijervine.[3]—Fraction 1-E (Table I) was dissolved in hot chloroform and filtered. On standing, a crystalline compound separated (needles, 15 mg.), m. p. 247–248° (decompn.), $[\alpha]_D^{28}$ +9° (c 0.68, pyr.) identified as rubijervine. For the authentic sample of rubijervine, $[\alpha]_D^{28}$ = +7° (c 1.01, pyr.). The infrared spectra of the rubijervine samples were determined in KBr pellets.

Component IX: Germidine.—A benzene solution of fraction 1-I (Table I) was chromatographed on Merck acid-washed alumina (4 Gm., 0.7-cm. diameter column). Elution with benzene-chloroform (75% chloroform to pure chloroform) yielded a crude product (25 mg.) consisting mainly of component IX. Crystallization from ether gave germidine [prisms, 5 mg., m. p. 235–237° (decompn.)].

Component X: Germerine.—Continued elution of the column described for the isolation of component IX with chloroform and chloroform-methanol

(from 0.5 to 5% methanol) yielded a crude product (40 mg.) consisting mainly of component X. Crystallization from ether-petroleum ether led to the isolation of germerine [prisms, 6 mg., m. p. 201.5–203.5° (decompn.)].

Germerine was also isolated from fraction 2-T (Table II). A benzene solution of fraction 2-T was chromatographed on Merck acid-washed alumina (1 Gm., 0.7-cm. diameter column). Elution with benzene-chloroform (1:1, 100 cc.) gave nonalkaloidal colored material. Further elution with chloroform-methanol (95:5, 100 cc.), evaporation of eluate to dryness and crystallization from ether-petroleum ether yielded germerine [prisms, 17 mg., m. p. 196–198° (decompn.)].

Component XI: Protoveratrine B.—A benzene solution of fraction 2-V (Table II) was chromatographed on Merck acid-washed alumina (1 Gm., 0.7-cm. diameter column). Elution with benzene led to the removal of colored material. Further elution with chloroform-methanol (98:2), evaporation of eluate to dryness and crystallization from ether led to the isolation of 4 mg. of protoveratrine B.

Evidence for Identification of Other Components.—*Component VII: Veratramine.*[a]—Paper chromatographic analysis of cryptenamine and of certain fractions thereof indicated the presence of a component with R_f identical with that of veratramine. The best separation of component VII was achieved with solvent system *A*.

Component XII: Germbudine.—Paper chromatographic analysis of fractions 1-K (Table I) and 2-W, 2-X, 2-Y (Table II) suggested the presence of a compound having an R_f identical with that of germbudine; it was best separated from other components with solvent system *B* (development period, twenty hours).

Component XIII: Neogermbudine.—Paper chromatographic analysis of fractions 1-K (Table I) and 2-W, 2-X, 2-Y (Table II) suggested the presence of a compound having an R_f identical with that of neogermbudine. Component XIII could also be best separated from other components with solvent system *B* (development period, twenty hours). Neogermbudine demonstrated a higher R_f than germbudine.

RESULTS AND DISCUSSION

As described above, cryptenamine was fractionated by partition and adsorption column chromatography; the progress of separations was followed by paper chromatographic analysis. By this procedure, during which cryptenamine was never subjected to conditions favorable for ester hydrolysis, the known (12, 13) hypotensive alkaloids protoveratrine A (IV), protoveratrine B (XI), neogermitrine (III), germitrine (VI), germidine (IX), and germerine (X) were isolated. In addition, it was possible to isolate the relatively nonhypotensive alkaloids (14) isorubijervine (I), jervine (V), rubijervine (VIII), and an apparently new noncarbonyl alkamine (II). Paper chromatographic evidence suggesting the presence of hypotensively active germbudine (XII) (15) and neogermbudine (XIII) (16) and of relatively inactive veratramine (VII) (14) was also obtained. It was estimated that the components listed represent some 80%, by weight, of cryptenamine (Tables I and II).

TABLE III.—HYPOTENSIVE PRINCIPLES OF ACTIVE FRACTIONS[a]

Fraction	Estimated % Characterized[b]	Hypotensive Alkaloids Identified	Total Hypotensive Units[c]
1-B	90	Protoveratrine A, neogermitrine	43,000
1-C	90	Neogermitrine, germitrine	198,000
1-D	100	Germitrine	88,000
1-H	40	Germidine	32,000
1-I	90	Germidine, germerine	35,000
1-J	70	Germidine, germerine, protoveratrine B	58,000
Total	80	...	454,000
Cryptenamine	80	...	500,000

[a] Fractions from partition chromatography (Table I) of 2 Gm. of cryptenamine.
[b] See footnote[d], Table I.
[c] Obtained by multiplication of weight of fraction by hypotensive units/mg. Cryptenamine assay, 250 hypotensive units/mg.

The pharmacological results summarized in Table III indicate that the six highly active fractions obtained on chromatographic separation of cryptenamine (Table I) represent approximately 90% of the total hypotensive activity of cryptenamine. The estimated proportion of total characterized components in these fractions is 80% and the active principles identified in these fractions represent the most powerful hypotensive alkaloids of *Veratrum viride* (7, 17). In view of the foregoing, it appears likely that known ester alkaloids are responsible for most of the hypotensive activity of cryptenamine.

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Veratrum Alkaloids XXXVII*

The Structure of Germanitrine, A Hypotensive Ester Alkaloid

By S. MORRIS KUPCHAN and ADRIANO AFONSO

The structure of germanitrine, a highly active hypotensive ester alkaloid, has been elucidated. Germine 3-angelate 7-acetate 15-*l*-2′-methylbutyrate, synthesized from germine, was found to be identical with germanitrine.

GERMANITRINE is a highly active hypotensive ester alkaloid isolated from *Veratrum fimbriatum* Gray (1). Alkaline hydrolysis of germanitrine has afforded the known alkamine germine (I) (2) and one mole equivalent each of acetic, tiglic, and *l*-2-methylbutyric acids (1). Methanolysis of germanitrine resulted in loss of the acetate grouping with conversion to the diester germanidine (1). The latter compound yielded one mole equivalent of tiglic acid and one mole equivalent of *l*-2-methylbutyric acid on alkaline hydrolysis. The positions of the esterifying acids on the germine nucleus have now been established, thereby effecting the elucidation of the complete structures of germanitrine and germanidine.

Tentative assignment of the acetate group of germanitrine to C_7 of the germine nucleus has been made on the basis of its reactivity toward methanolysis and its characteristically high contribution to the molecular rotation of the triester (3). A common feature of the naturally-occurring germine triesters of known structure is attachment of the three acyl moieties to positions C_3, C_7, and C_{15} (3, 4), and it appeared reasonable as a preliminary working hypothesis to assign the tiglate and *l*-2-methylbutyrate residues to C_3 and C_{15}. The similarity of the optical rotations of germanidine (1) to those of all other known C_3, C_{15} diesters of germine (3) supported a 3,15-diester structure for germanidine. Because only small comparison samples of germanitrine and germanidine were available to us, a degradative structure elucidation was precluded. Instead, a partial synthesis of germanidine and germanitrine starting with germine was undertaken.

The first objective selected was germine 3-tiglate 15-*l*-2′-methylbutyrate. Germine 15-*l*-2′-methylbutyrate (II) (4, 5) was treated with a limited amount of tigloyl chloride in pyridine to yield the desired diester III. The tiglate group of III was assigned to C_3 on the following bases. The diester was stable to periodic acid, an indication that no free 1,2-glycol system was present. This fact limited the possible sites of attachment of the tiglate residue to C_3 or C_4. The diester consumed two oxygen equivalents of chromic acid, which indicated that only two secondary hydroxyl groups were present and that the tiglate residue was therefore affixed at C_3. The infrared spectrum of germine 3-tiglate 15-*l*-2′-methylbutyrate (III) differed significantly from that of germanidine.

The next alternative explored was the hypothetical formulation of germanidine as a 3-*l*-2′-methylbutyrate 15-tiglate. Treatment of germine with a limited amount of tigloyl chloride in pyridine afforded germine 15-tiglate (IV). That the tiglate was affixed to C_{15} was shown by the periodic acid consumption of one mole equivalent. Only a C_{15} monoester of germine possesses a single 1,2-glycol system. Furthermore, the amorphous periodic acid cleavage product showed infrared absorption at 3.65, 5.62, and 5.80 μ characteristic of the aldehydo-γ-lactone resulting from cleavage of the ring A glycol (2). Germine 15-tiglate (IV) was treated with a limited amount of *l*-2-methylbutyryl chloride in pyridine to yield germine 3-*l*-2′-methylbutyrate 15-tiglate (V). Evidence for the 3,15-diester formulation V paralleled that for III. The infrared spectrum of V also differed significantly from that of germanidine.

The original proposal that the tiglate, rather than the geometrically isomeric angelate residue was present in germanitrine was based on the identification of *p*-phenylphenacyl tiglate among the *p*-phenylphenacyl esters derived from the acids formed upon hydrolysis (1). As a control, it was shown that neither pure angelic nor pure tiglic acid isomerized appreciably under the alkaline conditions employed for the hydrolysis of the ester alkaloids (1). On the other hand, it appeared reasonable that isomerization of angelate to tiglate might have occurred prior to or during hydrolysis of the ester alkaloids, or even during the preparation of the *p*-phenylphenacyl esters.

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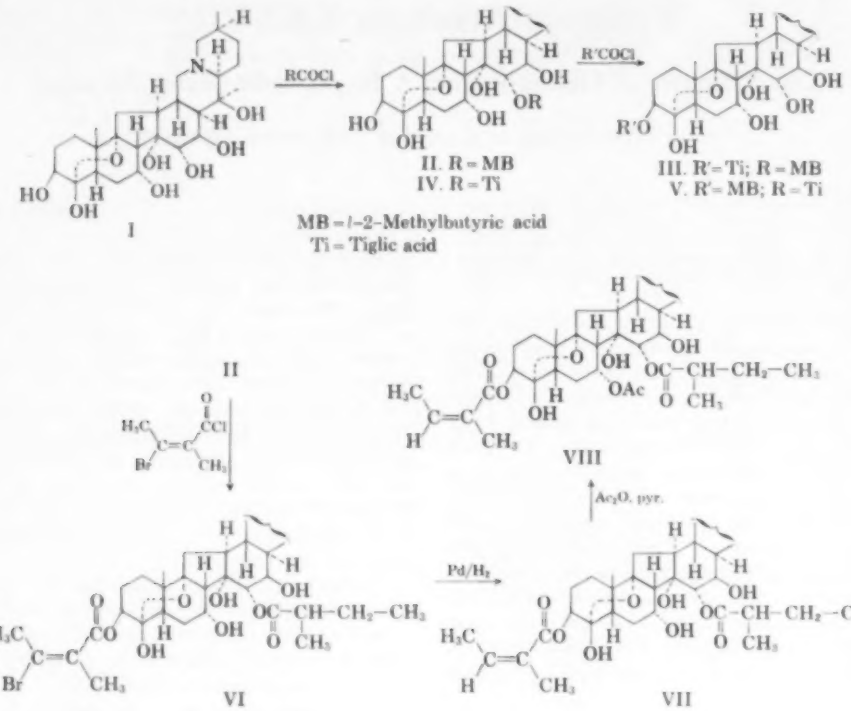

The historical differences concerning the acid residue present in the schoenocaulon ester alkaloid cevadine are best rationalized on the basis that isomerization of the angelate residue to tiglate may take place prior to or during hydrolysis. In the case of cevadine, some authors reported hydrolysis to tiglic acid (6, 7), some to angelic acid (8, 9, 10), and some to a mixture of angelic and tiglic acids (11).

A partial synthesis of germine 3-angelate 15-*l*-2′-methylbutyrate (VII) was next undertaken. Germine 15-*l*-2′-methylbutyrate (II) was treated with a limited amount of 3-bromoangeloyl chloride (12) to yield germine 3-(3′-bromoangelate) 15-*l*-2′-methylbutyrate (VI). The latter compound, on hydrogenolysis, afforded germine 3-angelate 15-*l*-2′-methylbutyrate (VII). Evidence for the 3,15-diester formulation VII paralleled that for III. The melting point of the synthetic diester was not depressed on admixture with an authentic sample of germanidine. The infrared spectra and paper chromatographic behavior of the respective samples were identical.

Acetylation of germanidine (VII) with a limited amount of acetic anhydride yielded the triester germine 3-angelate 7-acetate 15-*l*-2′-methylbutyrate (VIII). Evidence for the attachment of the acetate group at C_7 was obtained in the following manner. A chromic acid titration of the triester showed the presence of only one secondary hydroxyl group and C_4 was thereby excluded as a site for attachment of the acetate ester. Furthermore, the oxidation product, on alkaline treatment, afforded a diosphenol with ultraviolet spectral properties identical with those of the diosphenol from neogermitrone and 16-dehydrogermine 3,4,7,15-tetraacetate (3). The triester showed no depression in melting point on admixture with an authentic sample of germanitrine. The infrared spectra and paper chromatographic behavior of the respective samples were identical.

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Ultraviolet absorption spectra were determined in 95% ethanol on a Cary recording spectrophotometer (model 11 MS). Infrared spectra were determined in chloroform on a Baird double beam recording spectrophotometer. Microanalyses were carried out by Dr. S. M. Nagy and his associates at the Massachusetts Institute of Technology on samples dried under reduced pressure at 110°.

Periodic Acid Titrations.—These titrations were

performed as in Part XXVIII (2) except that one-third of the total volume of water was replaced with *t*-butanol.

Chromic Acid Titrations.—The method described in Part XXXII (4) was used. Results are summarized in Table I.

Paper Chromatography. The technique followed was that described by Levine and Fischbach (13). Unless otherwise specified the *n*-butyl acetate: *n*-butanol: formic acid (25:5:1 by volume) system was used.

Germine 3-Tiglate 15-l-2′-Methylbutyrate (III).—To germine 15-*l*-2′-methylbutyrate (5) (0.4 Gm.) in pyridine (8 cc.) cooled to 0°, was added tigloyl chloride (14) (0.9 Gm.) with stirring. The solution was kept at room temperature for twenty-four hours. The reaction mixture was then cooled by adding crushed ice, made alkaline (pH 8) with a 10% sodium carbonate solution, and extracted several times with chloroform. The chloroform extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was dissolved in benzene and the solution was evaporated to dryness. This procedure was repeated until all traces of pyridine had been removed. The amorphous residue was chromatographed on Merck acid-washed alumina (10 Gm.), and the progress of the column separation was followed with a paper chromatogram of each fraction collected. Elution with benzene (100 cc.) yielded a substance (90 mg.) having a high R_f, presumably a triester. Germine 3-tiglate 15-*l*-2′-methylbutyrate (III, 0.275 Gm.) was eluted with 50% benzene-chloroform (400 cc.). The diester III was crystallized from acetone-ether as pale yellow prisms (150 mg.), m. p. 227–228° (decompn.); $[\alpha]_D^{21} + 3°$ (c 2.65, pyr.).

Anal.—Calcd. for $C_{37}H_{57}NO_{10}$: C, 65.77; H, 8.44. Found: C, 65.39; H, 8.58.

In a volatile acid determination (15) 15.31 mg. of the compound yielded an amount of acid equivalent to 5.05 cc. of 0.009181 *N* sodium thiosulfate; calcd. for 1 mole equivalent of tiglic acid and 1 mole equivalent of *l*-2-methylbutyric acid as required by structure III, 4.94 cc.

Diester III did not consume any periodic acid after six and one-half hours. The infrared spectrum of III was not identical with that of germanidine.

Germine 15-Tiglate (IV).—To a cold (0°) solution of germine (3.2 Gm.) in pyridine (25 cc.) was added tigloyl chloride (14) (0.9 Gm.). The mixture was stirred until homogeneous, allowed to warm to room temperature and stand for twenty-four hours. Further treatment followed the procedure used for III. The amorphous reaction product (3.27 Gm.) was chromatographed on Merck acid-washed alumina (60 Gm.). The separation was followed with a paper chromatogram of each fraction collected, using the resolving system for slow-moving alkaloids (13) [1] Elution with chloroform (1,000 cc.) yielded a product having a high R_f, presumably a diester. Further elution with 3% methanol-chloroform afforded germine 15-tiglate (IV) (1.16 Gm.) which was crystallized from chloroform-acetone as colorless prisms (720 mg.), m. p. 224–225° (decompn.), $[\alpha]_D^{21} - 7°$ (c 2.15, pyr.).

[1] The solution was prepared by adding 1 cc. of formic acid to the separated organic layer of the system *n*-butyl acetate: *n*-butanol: water (10:25:10 by volume).

TABLE I.—CHROMIC ACID TITRATIONS

Ester	Oxygen Equivalents Consumed 1 hr.	4 hr.
III	1.8	2.3
V	1.6	1.9
VII	1.7	2.1
VIII	1.3	1.4

Anal.—Calcd. for $C_{32}H_{49}NO_9$: C, 64.95; H, 8.35. Found: C, 65.22; H, 8.24.

In a volatile acid determination (15) 15.46 mg. of the compound yielded an amount of acid equivalent to 2.00 cc. of 0.009181 *N* sodium thiosulfate; calcd. for 1 mole equivalent of tiglic acid as required for structure IV, 2.80 cc.

Monoester IV consumed 1.1 mole equivalents of periodic acid after one hour and was stable thereafter. The solution remaining from the periodic acid oxidation was made alkaline (pH 8) with dilute ammonia and extracted with chloroform. The chloroform extracts were dried over sodium sulfate and the amorphous residue left on evaporation showed infrared absorption at 3.65, 5.62, and 5.80 μ.

Germine 3-l-2′-Methylbutyrate (V).—Germine 15-tiglate (IV) (0.4 Gm.) in pyridine (4 cc.) was treated with *l*-2-methylbutyryl chloride (5) (0.116 Gm.) under the same conditions used for diester III. The amorphous reaction product was chromatographed on Merck acid-washed alumina (10 Gm.). Elution with benzene (100 cc.) yielded a product (0.12 Gm.) having a high R_f, presumably a triester. Germine 3-*l*-2′-methylbutyrate 15-tiglate (V) (0.2 Gm.) was eluted with 50% benzene-chloroform (540 cc.) and crystallized with difficulty from acetone, as colorless plates (55 mg.), m. p. 180–181° (decompn.); $[\alpha]_D^{21} + 3°$ (c 1.10, pyr.).

Anal.—Calcd. for $C_{37}H_{57}NO_{10}$: C, 65.77; H, 8.44. Found: C, 65.35; H, 8.70.

In a volatile acid determination (15) 8.0 mg. of the compound yielded an amount of acid equivalent to 2.70 cc. of 0.009181 *N* sodium thiosulfate; calcd. for 1 mole equivalent of tiglic acid and 1 mole equivalent of *l*-2-methylbutyric acid as required by structure V, 2.58 cc.

Diester V was stable toward periodic acid. The infrared spectrum of V was not identical with that of germanidine.

Germine 3-Angelate 15-l-2′-Methylbutyrate (VII).—To a solution of germine 15-*l*-2′-methylbutyrate (0.365 Gm.) in pyridine (5 cc.) was added with stirring, 3-bromoangeloyl chloride (12) (0.135 Gm.) at room temperature. The reaction mixture was allowed to stand for twenty-four hours and further treatment followed the procedure used for diester III. The reaction products from two such batches were combined (0.92 Gm.) and chromatographed on Merck acid-washed alumina (16 Gm.). Elution with benzene (250 cc.) yielded an amorphous product (0.07 Gm.) with a high R_f, presumably a triester. Elution with 50% benzene-chloroform (500 cc.) followed by chloroform (870 cc.) yielded germine 3-(3′-bromoangelate) 15-*l*-2′-methylbutyrate (VI) (0.312 Gm.) as a pale yellow amorphous material, $[\alpha]_D^{21} + 16°$ (c 1.05, ethanol).

To a suspension of 10% palladium on carbon (0.07 Gm.) in 95% ethanol (10 cc.) which had previously been saturated with hydrogen, was added a solution

of VI (0.14 Gm.) in ethanol (4 cc.) containing anhydrous sodium acetate (0.07 Gm.). After hydrogenation at atmospheric pressure for three minutes the theoretical uptake of one mole equivalent of hydrogen was complete and the hydrogenation was terminated. The catalyst was removed by filtration, washed with ethanol (15 cc.), and the filtrate was evaporated under reduced pressure. The residue was suspended in water (5 cc.), made alkaline (pH 8) with a 10% sodium carbonate solution, and extracted several times with chloroform. The chloroform extract was dried over anhydrous sodium sulfate. Evaporation under reduced pressure yielded germine 3-angelate 15-*l*-2′-methylbutyrate (0.130 Gm.) which was crystallized from acetone-water as colorless flat needles (115 mg.), m. p. 220–221° (decompn.), $[\alpha]_D^{23}$ −3° (c 1.20, pyr.). The melting point was not depressed on admixture with an authentic sample of germanidine.[2]

Diester VII was stable to periodic acid. The infrared spectra and paper chromatographic behavior of VII and an authentic sample of germanidine[2] were identical.

Germine 3-Angelate 7-Acetate 15-l-2′-Methylbutyrate (VIII).—To a solution of VII (0.120 Gm.) in pyridine (1 cc.) was added acetic anhydride (0.021 Gm.) at 0°. The reaction mixture was allowed to stand at room temperature for twelve hours. Excess acetic anhydride was destroyed with two drops of methanol and the mixture was evaporated under reduced pressure at room temperature. The residue was dissolved in benzene and evaporated to dryness. This procedure was repeated several times to remove traces of pyridine. The residue was dissolved in ice water (3 cc.), made alkaline (pH 8) with 10% sodium carbonate solution, extracted with chloroform, and the extract evaporated to dryness. The amorphous residue (0.120 Gm.) was dissolved in chloroform and petroleum ether (Skelly B) was added to the solution until an amorphous precipitate separated. The precipitate was removed by filtration and rejected. The filtrate on concentration deposited crystalline triester VIII (0.055 Gm.) which had the same R_f as germanitrine.[2] Recrystallization from acetone-water afforded colorless needles (50 mg.), m. p. 225–226° (decompn.). The melting point was not depressed on admixture with an authentic sample of germanitrine.[2] The infrared spectrum of triester VIII was identical with that of germanitrine.

Chromic Acid Oxidation of Germanitrine.—The chromic acid titrations of germanitrine (Table I) were performed on two 2-cc. aliquot parts taken from a solution (10 cc.) containing 29.1 mg. of germanitrine. The solution remaining after removal of the aliquots was treated with a sodium bisulfite solution [illegible]een, made alkaline with 20% NaOH, and extracted with chloroform. The extract was evaporated to dryness under reduced pressure. The residue was dissolved in 1% methanolic sodium hydroxide (4 cc.) and heated under reflux for twelve minutes. The deep red solution was acidified with glacial acetic acid, evaporated to dryness, made alkaline with dilute ammonia, and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness. The brown resin was treated with ether and the insoluble brown impurities were removed by filtration. The filtrate, on evaporation, yielded a pale yellow amorphous residue (6 mg.), $\lambda_{max.}$ 320 mμ (ε 11,200), 285 mμ (ε 6,400); $\lambda^{0.01\ N NaOH}_{max.}$ 375 mμ (ε 8,600), 343 mμ (ε 6,600).

[2] We thank Mr. Murle Klohs of Riker Laboratories, Northridge, Calif., for comparison samples of germanitrine and germanidine.

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Veratrum Alkaloids XL*

The Structure of Escholerine, A Hypotensive Ester Alkaloid

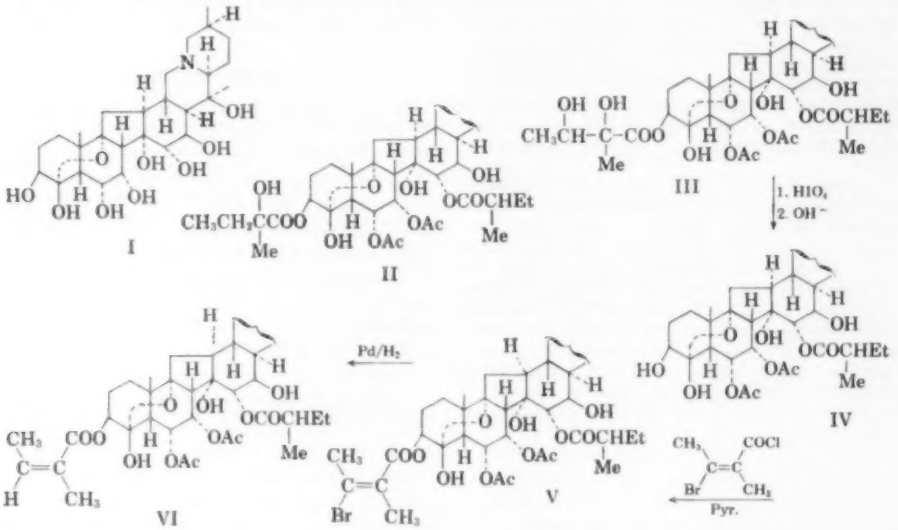
By S. MORRIS KUPCHAN and C. IAN AYRES

The structure of escholerine has been elucidated. Synthesis of protoverine 3-angelate 6,7-diacetate 15-*l*-2′-methylbutyrate afforded a product identical with escholerine.

ESCHOLERINE is one of the main hypotensive principles of *Veratrum eschscholzii* Gray (1, 2). Hydrolysis of escholerine has afforded the known alkamine protoverine (I) (3, 4, 5), two mole equivalents of acetic acid, one mole equivalent of *l*-2-methylbutyric acid and one mole equivalent of angelic acid (1, 2). The positions of the four esterifying acids on the protoverine nucleus have now been established, thereby effecting the elucidation of the complete structure of escholerine.

A common structural feature of the two known naturally-occurring esters of protoverine, protoveratrine A (II), and protoveratrine B (III) is the attachment of acetates at positions C_6 and C_7 and of *l*-2-methylbutyrate at C_{15} (6, 7). Consequently, on a tentative biogenetic basis, one might expect a similar distribution of these three acids in escholerine. Furthermore, all the hypotensive protoverine esters studied to date possess a free (unacylated) hydroxyl group at C_{16}. Hence to test this hypothesis, the synthesis of protoverine 3-angelate 6,7-diacetate 15-*l*-2′-methylbutyrate (VI) was undertaken.

Protoverine 6,7-diacetate 15-*l*-2′-methylbutyrate (IV) can be readily prepared from protoveratrine B (7). Triester IV was treated with a limited amount of 3-bromoangeloyl chloride (8, 9) to yield protoverine 3-(3′-bromoangelate) 6,7-diacetate 15-*l*-2′-methylbutyrate (V) $[\alpha]_D^{25}$ −16° (pyr.). The latter compound, on hydrogenation, afforded protoverine 3-angelate 6,7-diacetate 15-*l*-2′-methylbutyrate (VI), m. p. 235–236° (decompn.) $[\alpha]_D^{26}$ −28° (pyr.). The angelic acid was shown to be attached to C_3 in the following manner. A chromic acid titration of the tetraester showed the presence of only one secondary hydroxyl group and C_4 was thereby excluded as a site for attachment of the angelic acid ester. Furthermore, the oxidation product, on treatment with alkali, afforded a diosphenol with spectral properties identical with those of the diosphenol from 16-dehydroprotoverine 3,4,6,7,15-pentaacetate (5). Therefore, the synthetic tetraester was protover-



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Part XXXIX in the Series; Kupchan S. M., and Ayres, C. I., *J. Am. Chem. Soc.*, in press.

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ine 3-angelate 6,7-diacetate 15-*l*-2′-methylbutyrate (VI). The compound showed no depression in melting point on admixture with an authentic sample of escholerine. The infrared spectra and paper chromatographic behavior of the respective samples were identical.

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Ultraviolet absorption spectra were determined on a model 11 MS Cary recording spectrophotometer and 95% ethanol was used as solvent. Infrared spectra were determined on a Baird model B double beam infrared recording spectrophotometer and chloroform was used as a solvent.

Protoverine 3-(3′-bromoangelate) 6,7-diacetate 15-*l*-2′-methylbutyrate (V).—Protoverine 6,7-diacetate 15-*l*-2′-methylbutyrate [(7), 627 mg., m. p. 232–233° (decompn.)] was dissolved in pyridine (10 cc.), cooled to −10°, and treated with 3-bromoangeloyl chloride (0.2 cc.). The mixture was stirred until a clear yellow solution was obtained. The solution was allowed to come to room temperature and stand overnight. The pH was brought to 9 with dilute ammonia and the mixture extracted thoroughly with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to yield a brown resin (640 mg.). A paper chromatogram employing the procedure of Levine and Fischbach (10) [*n*-butyl acetate: *n*-butanol: formic acid (25:5:1 by volume)] indicated the formation of a new material in a yield of approximately 25%. The resin was chromatographed on Merck acid-washed alumina (15 Gm.). The column yielded to benzene, benzene-chloroform, and chloroform a series of yellow oils. When developed with chloroform-1% methanol, however, an amorphous material was obtained (110 mg.), $[\alpha]_D^{23}$ −16° (c 0.89, pyr.) whose paper chromatogram indicated that it was homogeneous in nature.

Hydrogenation of Protoverine 3,(3′-bromoangelate) 6,7-diacetate 15-*l*-2′-methylbutyrate: Synthesis of Escholerine (VI).—The amorphous 3,(3′-bromoangelate) (67 mg. of V. $[\alpha]_D^{23}$ −16°) was dissolved in 95% ethanol (6 cc.) containing sodium acetate (45 mg.) and added to a suspension of 10% palladium on charcoal (35 mg.) in 95% ethanol (4 cc.) that had previously been saturated with hydrogen. After two minutes the theoretical uptake of hydrogen was consumed and the hydrogenation was terminated. The catalyst was removed by filtration and the filtrate was acidified with glacial acetic acid and evaporated nearly to dryness. The residue was dissolved in water, basified with dilute ammonia, and extracted with chloroform. The chloroform solution was dried over anhydrous sodium sulfate and evaporated to yield a resin which was crystallized from acetone-water to give colorless plates, m. p. 235–236° (decompn.); $[\alpha]_D^{26}$ − 28° (c 0.9, pyr.); yield, 30 mg. The melting point was not depressed on admixture with an authentic sample of escholerine.[1] The infrared spectra and paper chromatographic behavior of the respective samples were identical. A second crop of material (15 mg.) with m. p. 234–236° (decompn.) was also obtained.

Chromic Acid Oxidation of Escholerine.—Protoverine 3-angelate 6,7-diacetate 15-*l*-2′-methylbutyrate (escholerine, m. p. 234–236°, 15 mg.) in glacial acetic acid (5 cc.) was treated with 0.05 *N* chromic anhydride in 99.8% acetic acid (3 cc.). The solution was allowed to stand at room temperature. Aliquot parts titrated after one hour and three hours showed the consumption of 1.20 and 1.28 oxygen equivalents of chromic acid. An aliquot (4 cc.) taken after one hour was treated with excess sodium bisulfite to destroy the oxidizing agent, basified with dilute ammonia, and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to give a resin (7 mg.). The resin was dissolved in 2 cc. of a solution of sodium hydroxide (100 mg.) in methanol (10 cc.) and heated under reflux for twelve minutes. The reaction mixture was acidified with glacial acetic acid and evaporated to dryness. The residue was dissolved in water, basified with dilute ammonia, and extracted with chloroform. Evaporation of the chloroform afforded an amorphous residue which showed $\lambda_{max.}$ 328 mμ (ε 12,700) and $\lambda_{max.}^{0.1\ N\ NaOH}$ 381 mμ (ε 8,800).

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[1] We thank Mr. Murle Klohs of Riker Laboratories, Inc., Northridge, Calif., for a comparison sample of escholerine.

Veratrum Alkaloids XLI*

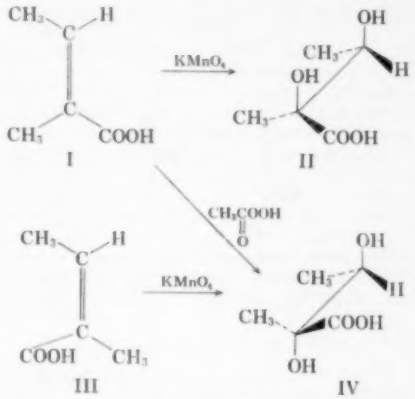
The Structure of Germbudine, A Hypotensive Ester Alkaloid

By S. MORRIS KUPCHAN and NORBERT GRUENFELD†

The structure of germbudine has been elucidated as germine 3-*d-threo*-2′,3′-dihydroxy-2′-methylbutyrate 15-*l*-2 -methylbutyrate (V). Oxidation of germbudine with periodic acid and subsequent alkaline hydrolysis afforded germine 15-*l*-2′-methylbutyrate (VIII).

GERMBUDINE is a minor hypotensive constituent of alkaloidal extracts of *Veratrum viride* (1). Alkaline hydrolysis of germbudine was reported to yield the known alkamine germine (IX), one mole equivalent of 2-methylbutyric acid, and one mole equivalent of high melting *d*-2,3-dihydroxy-2-methylbutyric acid.

Low melting *l*-2,3-dihydroxy-2-methylbutyric acid is one of the esterifying acids of neogermbudine and germitetrine, and high melting *d*-2,3-dihydroxy-2-methylbutyric acid occurs in germbudine and protoveratrine B (1–5). In 1953, Nash and Brooker (3) suggested that these two acids were diastereoisomers on the basis of their differing physical characteristics and the identification of acetaldehyde and pyruvic acid as common periodic acid cleavage products. In 1955, Myers, *et al.* (1), confirmed this hypothesis by synthesis of both acids. The racemic low melting diastereoisomer was synthesized by hydroxylation of tiglic acid (I) with dilute permanganate and was resolved to obtain the *l*-2,3-dihydroxy-2-methylbutyric acid identical with the acid isolated from natural sources (*levo* II). Hydroxylation of a double bond with permanganate is known to proceed in a *cis* fashion (6) and therefore the low melting diastereoisomer is the *erythro* isomer (II). The racemic high melting diastereoisomer was synthesized by hydroxylation of tiglic acid with peracetic acid and was not resolved. However derivatives of the racemic compound and dextrorotatory natural acid showed identical infrared spectra. The natural acid also had a melting point different from that of the synthetic *d-erythro acid*. Since peracetic acid is known to hydroxylate a double bond in a *trans* fashion (6) the high melting diastereoisomer is the *threo* isomer (IV). This assignment is supported by the observation that the latter racemic synthetic acid has the same melting point as the acid obtained on hydroxylation of angelic acid (III) with dilute permanganate (7).



Thus the esterifying acid of neogermbudine and germitetrine is *l-erythro*-2,3-dihydroxy-2-methylbutyric acid and the esterifying acid of germbudine and protoveratrine B is *d-threo*-2,3-dihydroxy-2-methylbutyric acid. These assignments render the chemical nomenclature of these hypotensive alkaloids more meaningful than the one based on melting points of the acids (1, 2, 8).

A common structural feature of most naturally-occurring diesters of germine is the attachment of the acyl groups at C_3 and C_{15}. Neogermbudine, which occurs alongside germbudine in *Veratrum viride*, has been shown to be germine 3-*l-erythro*-2′,3′-dihydroxy-2′-methylbutyrate 15-*l*-2′-methylbutyrate (VI) (2). Germbudine and neogermbudine possess very similar melting points, optical rotations, and infrared spectra (1). They also demonstrate very similar paper chromatographic behavior. The ethylene chloride-cellosolve acetate-pyridine system of Levine and Fischbach (9) proved adequate for their separation, but only after prolonged development.

On the basis of the similarity of physical constants, the possibility that germbudine could be a diastereoisomer of neogermbudine was formulated as a working hypothesis for the elucidation of its structure. Germbudine, in agreement with the postulation of a C_3, C_{15} diester and the consequent presence of only one free glycol group (that of the dihydroxy acid) consumed 0.85 mole

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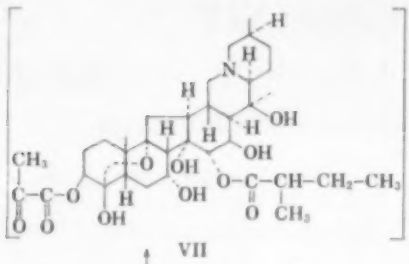

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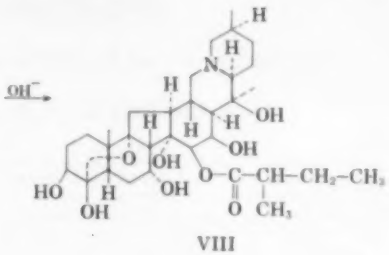

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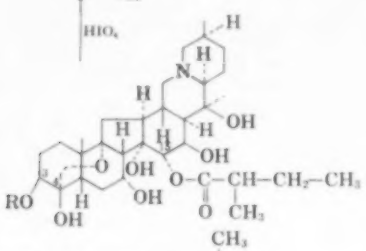
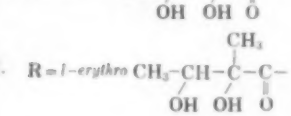
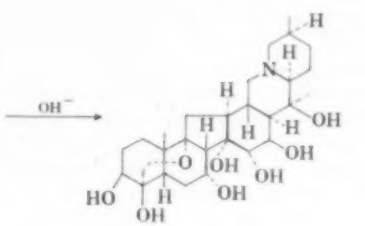


V. R = *d-threo* $CH_3-CH(OH)-C(OH)(CH_3)-C(=O)-$



VI. R = *l-erythro* $CH_3-CH(OH)-C(OH)(CH_3)-C(=O)-$



IX

equivalent of periodic acid. The periodic acid titer did not distinguish between the C_3, C_{18} and the C_4, C_{18} diester structures. The presence of an acyl moiety at C_4 was deemed highly improbable on biogenetic grounds. No C_4 ester has been found among the more than twenty naturally-occurring derivatives of the veratrum alkamines veracevine, germine, protoverine, and zygadenine (2, 8, 10–14). Evidence in favor of the C_3, C_{18} structure was obtained by chromic acid titration of the diester. Germbudine demonstrated a chromic acid consumption which paralleled that of neogermbudine and indicated the presence of three secondary hydroxyl groups.

The specific location of each acyl group was determined by the selective removal of the *d-threo*-2,3-dihydroxy-2-methylbutyryl moiety by a procedure similar to that employed for the structure elucidation of protoveratrine B (13). Oxidation of germbudine with periodic acid at room temperature for three hours gave, as determined by paper chromatographic analysis, a quantitative yield of a product of higher R_f than the starting material [presumably germine mono-2-methylbutyrate mono-pyruvate (VII)]. Taking advantage of the facile base-catalyzed hydrolysis of pyruvate esters (13, 15), the periodic acid oxidation mixture was rendered alkaline with ammonia after decomposition of excess periodic acid with sodium bisulfite and was kept at room temperature for twenty minutes. Paper chromatographic analysis of the chloroform extracted product revealed partial conversion to a compound having the same R_f as germine-15-*l*-2′-methylbutyrate (VIII) (2, 16). Incomplete hydrolysis may have been due to the formation of a bisulfite addition compound at the ketone function of the pyruvate. Retreatment of the product with dilute ammonia led to complete hydrolysis. Crystallization from chloroform-ether gave a product shown by infrared spectrum, paper chromatographic, and melting point behavior to be germine 15-*l*-2′-methylbutyrate. Hence the 2-methylbutyric acid of germbudine is assigned the *levo* configuration, and germbudine is germine 3-*d-threo*-2′,3′-dihydroxy-2′-methylbutyrate 15-*l*-2′-methylbutyrate (V).

The degradation of neogermbudine by a sequence of steps paralleling that described for germbudine also gave germine 15-*l*-2′-methylbutyrate. This route was found to be more elegant than the sodium borohydride method employed earlier (2).

EXPERIMENTAL

Melting points are corrected for stem exposure. Infrared spectra were determined on a Baird model B double beam infrared recording spectrophotometer and chloroform was used as the solvent. Paper chromatograms were run by the descending technique employing Whatman No. 1 paper.

Paper Chromatography.—The procedure and solvent systems employed were similar to those described by Fischbach and Levine (9, 17) for an ascending technique. This method involves the use of prewetted pH 3.5 buffered paper and the detection of alkaloids by spraying with a chloroform solution of bromphenol blue. The solvent systems used were: (*A*) the solution prepared by adding 1 cc. of formic acid to the separated organic layer of the system *n*-butyl acetate-*n*-butanol-water (10:25:10); (*B*) cellosolve acetate-ethylene chloride-pyridine (10:15:1). System *B* was used for testing the homogeneity of germbudine,[1] neogermbudine,[1] and germine - 15 - *l* - 2′ - methylbutyrate. Separation of these alkaloids was successful on development for eighteen hours allowing solvent to run off the paper, as R_f values were relatively low. System *B*, development period of twelve hours, was utilized to follow periodic acid oxidations and degradations of germbudine and neogermbudine. System *A* was employed for differentiation of germine 15-*l*-2′-methylbutyrate and germine 3-*l*-2′-methylbutyrate (protoveratridine) (8).

Degradation of Germbudine to Germine 15-*l*-2′-Methylbutyrate.—Germbudine (V, 21 mg.), m. p. 159–161°, was dissolved in 5% acetic acid (5 cc.); 0.05 *M* periodic acid (15 cc.) and sufficient distilled water to dilute the solution to 25 cc. were added. The periodic acid consumption, as determined by titration of 1-cc. aliquots (18), was 0.85 mole equivalent after one and after three hours. The remainder of the solution was first treated with 7% sodium bisulfite (10 cc.) and then with sufficient 2 *N* ammonium hydroxide to bring the solution to pH 8.5–9. After standing at room temperature for twenty minutes, the ammoniacal solution was extracted with chloroform; the combined chloroform solution was washed with water, dried with sodium sulfate and evaporated to dryness under reduced pressure. The paper chromatogram of the reaction product revealed the absence of starting material; it also suggested the presence of germine 15-*l*-2′-methylbutyrate (VIII) and of a second product of R_f higher than that of germbudine [possibly germine 3-pyruvate 15-*l*-2′-methylbutyrate (VII)]. The reaction product was dissolved in 5% acetic acid (5 cc.) and a few drops of chloroform; sufficient 2 *N* ammonium hydroxide was added to bring the solution to pH 9. The mixture was kept at room temperature for twenty minutes and then extracted with chloroform. The chloroform extract was washed, dried, and evaporated to dryness to give a product (15 mg.) which proved homogeneous on paper chromatographic analysis and showed R_f identical to that of germine 15-*l*-2′-methylbutyrate (VIII). The crude product was dissolved in a drop of chloroform, excess ether was added, and the flocculent precipitate which formed immediately was removed by filtration. White elongated prisms (9.5 mg.) separated from the filtrate on standing overnight and were recrystallized twice from chloroform-ether, yield 5 mg., m. p. 222–224° (decompn.). No melting point depression was observed on admixture with an authentic sample of germine 15-*l*-2′-methylbutyrate and the infrared spectra of the respective samples were identical (2, 16). The paper chromatographic behavior of the product was the same as that of the authentic sample and different from that of protoveratridine, germine 3-*l*-2′-methylbutyrate (8).

Degradation of Neogermbudine to Germine 15-*l*-2′-Methylbutyrate.—Neogermbudine (VI, 19.4 mg.), m. p. 149–152° (decompn.), was submitted to the degradation procedure described for germbudine. It showed a periodic acid uptake of 1.0 mole equivalent after one hour and 1.1 mole equivalents after three hours. Workup of the periodic acid oxidation mixture led to the isolation of germine 15-*l*-2′-methylbutyrate (VIII); first crop, 2.5 mg., m. p. 220–222° (decompn.); second crop, 5 mg., m. p. 215–218° (decompn.).

Chromic Acid Titrations.—The alkaloid (*ca.* 1 × 10^{-3} m*M*) was dissolved in glacial acetic acid (3 cc.) and treated with a solution of chromic anhydride in acetic acid containing 0.2% of water (2 cc., 0.055 *N*). The oxidation was allowed to proceed at room temperature and 2-cc. aliquots were withdrawn after reaction periods of one and two hours. The chromic acid in oxidation mixtures and blank was reduced with 10% potassium iodide solution (5 cc.) and the released iodine was titrated with standard 0.01 *N* sodium thiosulfate. The results of titrations run on identical molar concentrations of germbudine, neogermbudine, and germine 15-*l*-2′-methylbutyrate are given in Table I.

TABLE I.—CHROMIC ACID TITRATIONS

Alkaloid	Oxygen Equivalents of Chromic Acid Consumed Theoretical	Found, 1 hr.	Found, 2 hr.
Germine 15-*l*-2′-methylbutyrate (VIII)	3	3.0	3.3
Neogermbudine (VI)	3	2.9	3.3
Germbudine (V)	3	3.0	3.3

[1] We thank Dr. G. S. Myers of Ayerst, McKenna and Harrison Ltd. for authentic samples of germbudine and neogermbudine.

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An Improved Colorimetric Method for the Determination of Meprobamate in Biological Fluids*

By ALLAN J. HOFFMAN† and B. J. LUDWIG

A colorimetric procedure is described for the microdetermination of meprobamate in biological fluids. Meprobamate is extracted from plasma or serum using mixed chloroform-carbon tetrachloride solvent, and color development is effected by treatment with *p*-dimethylaminobenzaldehyde and antimony trichloride in acetic anhydride. The color intensity measured at 550 mμ is proportional to the concentration over the range of 0.5 to 10 mcg. A suitable modification of the extraction procedure for use with urine specimens is also described, which permits separation of meprobamate from the higher concentration of endogenous interfering substances present in urine. Determination of meprobamate concentrations in blood specimens from normal adult subjects indicates that a peak concentration of this drug is attained about two hours after oral administration.

IN EARLIER PUBLICATIONS (1–7) it has been shown that orally administered meprobamate is readily absorbed into the blood stream from the gastrointestinal tract of experimental animals and of humans and is excreted in the urine in an unchanged form, as a hydroxylated derivative, and in the form of a yet unidentified glucuronide. The analytical methods used in connection with these studies generally employed nonspecific colorimetric procedures or depended solely or partly on chromatographic separation. In some animal studies C^{14}-labeled meprobamate was utilized as a basis for analysis. None of the published methods permits a rapid and practical estimate of meprobamate content in blood and urine in the concentrations normally encountered and in the presence of endogenous interfering substances.

Ludwig and Hoffman (8) described a chemical reaction between meprobamate and certain aldehydes which served as a basis for a colorimetric procedure for the determination of this drug. It is the purpose of this publication to present a simplified and improved modification of this highly specific method and to show its application to a series of blood concentration and urinary excretion studies conducted on adult human subjects.

Meprobamate develops an intense red-violet color when reacted with certain aldehydes and anhydrous metallic salts in the presence of acid dehydrating agents.[1] An examination of the action of a number of such combinations led to the selection of *p*-dimethylaminobenzaldehyde and antimony trichloride in acetic anhydride as the reagents of choice from the viewpoint of prompt and consistent color development, permanence of color intensity, and adherence to Beer's law. This color reaction is specific for unsubstituted amides. By proper selection of extraction solvent interference from naturally-occurring amides, urea, etc., can be reduced to an insignificant minimum. Because of the greater water solubility of both hydroxymeprobamate and the glucuronide conjugate of this drug, the extraction procedure as described does not remove these metabolic products of meprobamate from the usual body fluids. When carried out according to the procedure given below, this method will permit the accurate determination of meprobamate in urine, plasma, serum, or spinal fluid in concentrations from 0.5 to 10 mcg. per ml.

EXPERIMENTAL

Reagents and Apparatus.—Reagent grade materials are used throughout and all solvents are redistilled through a packed column before use. Low actinic containers fitted with glass stoppers or Teflon-lined closures are recommended.

Antimony Trichloride-Acetic Anhydride Reagent (ATA Reagent).—A stock solution consisting of a saturated solution of antimony trichloride in chloroform (approximately 25% w/v) is first prepared by warming the mixture on a hot plate to solution, cooling, and filtering through Whatman 41H paper. ATA reagent is prepared by combining four volumes of this stock solution with one volume of acetic anhydride. This reagent is stable for two days when stored under refrigeration.

***p*-Dimethylaminobenzaldehyde Solution (DMB Reagent).**—A 1% w/v solution of the aldehyde in benzene. When stored in the refrigerator, this solution is stable for about one month.

Acetic Acid-Acetone Reagent (AAA Reagent).—A mixture of three volumes of reagent grade acetone and one volume of glacial acetic acid.

Mixed Solvent.—A mixture of equal volumes of carbon tetrachloride and chloroform.

Procedure.—One milliliter of plasma or serum is transferred to a 40-ml. glass-stoppered centrifuge tube. Two drops of 28% ammonium hydroxide, 2

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The authors wish to acknowledge the valuable technical assistance of Mr. John Gatto of these laboratories.

† Present address: White Laboratories, Inc., Kenilworth, N. J.

[1] The nature of the colored compound has not been investigated. Since the intense color development characteristic of this reaction apparently requires the participation of certain cations in the form of their anhydrous halides ($SbCl_3$, $SiCl_4$, $ZnCl_2$, etc.) it is unlikely that the colored compound possesses the simple anil structure postulated by Foley, Sanford, and McKennis (9) for a similarly colored compound formed from aniline and furfural.

drops of saturated potassium chloride solution, and 25 ml. of mixed solvent are added. A reagent blank consisting of 1 ml. of distilled water, ammonium hydroxide, potassium chloride solution, and mixed solvent is run simultaneously. The tubes are shaken vigorously for five minutes, then centrifuged for ten minutes. The organic layer is then carefully removed with a 30-ml. hypodermic syringe fitted with a 10-cm. needle and passed into a second centrifuge tube through a small pledget of Pyrex glass wool (previously extracted with chloroform). The glass wool is rinsed with 5 ml. of mixed solvent and the combined extract and rinse solutions evaporated to dryness by immersing the tube in an oil bath at 80°. The tubes are rinsed down with a few ml. of acetone and the heating continued until the tubes are thoroughly dried. Color development is then effected by adding 0.2 ml. of AAA reagent followed by 0.2 ml. of DMB reagent and mixing. One ml. of ATA reagent is pipetted into the tubes, the contents mixed, the tubes stoppered and placed immediately in a 50° water bath, and held at this temperature for exactly ten minutes. The tubes are removed and cooled by immersing in cold water and then diluted with 1 ml. of benzene. The colored solutions are transferred to Corex glass-stoppered absorption cells and the absorbance measured using a Beckman DU spectrophotometer at a wavelength of 550 mμ. A graph of the absorbance as a function of wavelength is shown in Fig. 1. Solutions of meprobamate in distilled water carried through the complete procedure as described are used to establish the calibration curve. The absorbance is directly proportional to the concentration over the range of 0.5–10 mcg. of meprobamate. Color intensity remains constant for at least one hour.

Conformity with Beer's Law.—A solution of meprobamate in water was prepared and aliquots containing 0, 2, 5, and 10 mcg. were diluted to 1 ml. with distilled water in one series and with normal human plasma in another series and treated in accordance with the above procedure. The absorbances of the colored solutions were read at 550 mμ against the prepared blank and were plotted against their respective concentrations (Fig. 2). The absorbances of solutions containing 0 to 10 mcg. of meprobamate gave a straight line relationship indicating conformance with Beer's law. The agreement between the distilled water and the plasma values at each concentration established that interfering components in plasma had been satisfactorily eliminated using this extraction procedure.

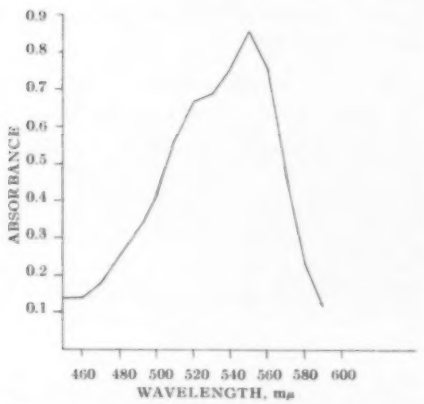

Fig. 1.—Absorption spectrum of meprobamate, *p*-dimethylaminobenzaldehyde and antimony trichloride color reaction. Determined with Beckman DU spectrophotometer using 1-cm. Corex cells, slit width 0.025 mm.

Similar studies made on human serum resulted in comparable recoveries of meprobamate and the procedure without modification was found to be suitable for spinal fluid studies. The presence of hemoglobin in plasma or serum had no adverse effect on this analytical method.

Accuracy and Precision.—To establish the accuracy and precision of this procedure, eight determinations were made at each of three concentrations in both water and normal human plasma. The precision at each level of concentration for each series is given in Table I.

TABLE I.—RECOVERY OF MEPROBAMATE FROM WATER AND HUMAN PLASMA

Meprobamate Added mcg./ml.	Absorbance, 550 mμ (Mean of 8 Determinations) Water	Plasma	Standard Deviation SD = $[1/N(x-\bar{x})^2]^{1/2}$ Water	Plasma
2.0	0.158	0.156	0.0127	0.0155
5.0	0.371	0.379	0.0158	0.0166
10.0	0.734	0.741	0.0280	0.0305

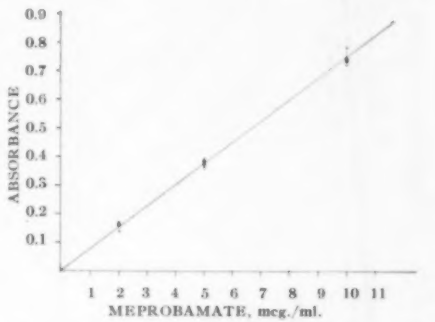

Fig. 2.—Absorbance of meprobamate, *p*-dimethylaminobenzaldehyde and antimony trichloride color reaction as a function of concentration: ○ water; ● plasma. Vertical lines denote magnitude of standard deviation for plasma determinations.

Analysis of Urine Specimens.—Urines of subjects receiving meprobamate collected during the twenty-four-hour period following administration generally contain meprobamate in amounts several-fold times the blood concentration of this drug, necessitating a 1:5 or 1:10 dilution prior to extraction. These dilutions contain interfering amounts of urea and other substances, and must be subjected to an additional treatment step before color development is attempted.

The residue obtained on evaporation of the

clarified mixed solvent extract is heated for fifteen minutes at 50° with 1 ml. of 0.2 *N* hydrochloric acid. It is then made alkaline with ammonia, potassium chloride solution added, and the procedure carried through as described above.

Application to Blood and Urine Specimens—Six healthy human adults were administered two 400-mg. tablets of meprobamate and blood specimens taken at one, two, four, six, eight, and twelve hours were analyzed for their meprobamate content. The individual values and a curve representing an average are plotted in Fig. 3. The peak blood concentrations occurred at about two hours, followed by a steady decline for ten hours or longer. At peak concentration, it is estimated that the total content of meprobamate in blood of these adult human subjects was about 15% of the ingested dose.

The meprobamate content of urines from several normal adult humans ingesting similar quantities of this drug were determined using the procedure as modified for urine analysis. In general, approximately 10% of the drug was excreted unchanged in the urine during the twenty-four-hour period following ingestion and a few additional per cent was excreted in the subsequent twenty-four-hour period. These findings are in agreement with those noted by others (1, 4, 5).

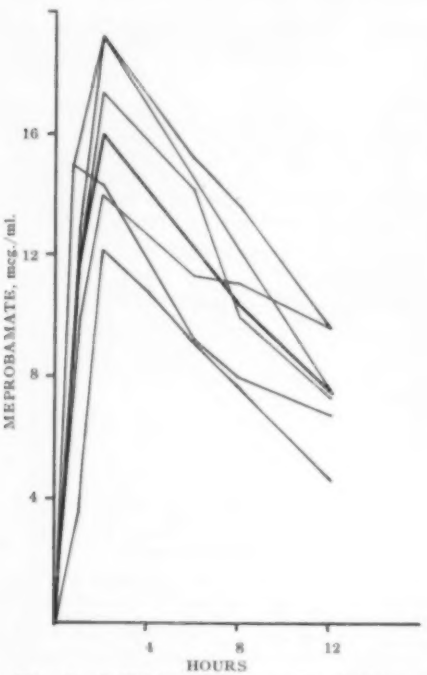

Fig. 3.—Individual plasma concentrations of meprobamate for adult human subjects receiving two 400-mg. tablets. Heavy line represents average of six individual studies.

DISCUSSION

A comparison of these human blood concentration values with those of experimental animals is of interest. Laboratory mice receiving substantially larger oral doses of meprobamate (300 mg./Kg.) showed a peak blood content of 245 mcg./ml. about one-half hour after oral administration. This result is consistent with the findings of Walkenstein, *et al.* (4), who reported that the peak C^{14} content of lung, liver, heart, spleen, and kidney occurred thirty minutes after oral administration of C^{14}-labeled meprobamate (400 mg./Kg.). In each case the rate of disappearance of meprobamate approximated that observed in humans. Whether this difference in time required to attain peak blood concentration is a function of the magnitude of the dose administered, or represents a significant species difference has not been elucidated.

A number of blood specimens from subjects who had consumed quantities of meprobamate far in excess of that usually prescribed were found to contain 100 to 200 mcg./ml. One such patient who allegedly consumed 12 Gm. of this drug had serum and spinal fluid levels of 182 and 50 mcg./ml., respectively. The urine of this patient contained 0.25% meprobamate.

SUMMARY

Meprobamate reacts with *p*-dimethylaminobenzaldehyde and antimony trichloride in the presence of acetic anhydride to give a red-violet color having an absorption maximum at 550 mμ. Concentrations of meprobamate in biological fluids have been accurately determined by use of a suitable extraction procedure and application of this color reaction.

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A Pharmacognostical and Phytochemical Study of *Maianthemum Canadense* (Desf.)*

By C. W. DICKERSON†

A number of valuable plant constituents have been obtained from the Lily family and, therefore, *Maianthemum Canadense* (Desf.) was deemed worthy of investigation. The taxonomy, distribution, and habitat of *Maianthemum Canadense* (Desf.) are recorded herein. A study of the morphological and histological features of the leaf, root, and whole plant powder, and the phytochemical and pharmacological properties of whole plant extracts are also described.

EXAMINATION of the scientific literature pertaining to *Maianthemum Canadense* (Desf.) revealed that the pharmacognostical, phytochemical, and pharmacological properties of this plant have not been recorded.

The plant was originally described by Réne Desfontaines, who gave it the name *Maianthemum*, derived from the Latin, Maius meaning May; and the Greek, anthemon meaning flower (1). The available information does not show any chemical analysis of *Maianthemum Canadense* (Desf.), but in 1907 Eldridge and Liddle (2) published their phytochemical study of *Smilacina racemosa* (Desf.), which closely resembles *Maianthemum*. Their results were as follows: the berries contain tartaric and oxalic acids, possibly in the form of potassium salts; tannin; levulose; and also some dextrose. The seeds contain levulose; fixed oil in the form of olein and palmitin; and some gum.

Fig. 1.—Flowering plants of *Maianthemum Canadense* (Desf.).

TAXONOMY

Maianthemum Canadense (Desf.) is a member of the family *Liliaceae* (Lily family). The most widely used synonym is wild lily-of-the-valley along with other common names of Canada mayflower and bead ruby, which are recorded by E. H. Hausman (3). According to A. B. Lyons (4) it is classified as follows: *Unifolium Canadense* (Desf.) Green (*Maianthemum Canadense* (Desf.) and *Smilacina bifolia*, var. *Canadensis* A. Gray). N. L. Britton, and A. Brown (5), and M. L. Fernald (1) also give false or wild lily-of-the-valley and two-leaved Solomon's seal as the common names. The Index Kewensis (6) records several names that have been given in the past: *Bifolium* by Gaertn., Mey, and Scherb.; *Maia* by Salisb.; *Sciophila* by Wibel; *Styandre* by Raffin.; and *Unifolium* by Hall., Enum., Stirp., and Helv.

MORPHOLOGY

The description of the plant found in Mecosta County, Big Rapids, Michigan, follows the description given in Gray's Manual of Botany (1), "extensively creeping, the freely-forking filiform rhizomes bearing stalked tuberous enlargements; a single cordate leaf accompanying the flowering stem; the latter erect, glabrous, 0.5–2.5 dm. high, bearing two or three glabrous leaves; lower cauline leaf 2–10 cm. long; flowers sweetly fragrant, the perianth segments about 2 mm. long." See Fig. 1.

DISTRIBUTION AND HABITAT

Maianthemum Canadense (Desf.) is found abundantly in wooded areas and recent clearings, in sandy humus soil. It often ascends to the subalpine areas of Labrador, south to Newfoundland, New England,

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Long Island, Delaware, Pennsylvania, upland to Georgia, Tennessee, and Iowa.

In Michigan it is widely distributed and found in moist wooded areas throughout the upper and lower peninsula.

EXPERIMENTAL

Leaf.—Two methods of microscopical study were used. The use of a microtome was employed and the usual preparation of the specimen was followed. The mountings obtained by the microtome method were not satisfactory for drawing purposes, therefore fresh cuttings were made and mounted in chloral hydrate water. These cuttings were observed at 10× and 43×.

The leaf of *Maianthemum Canadense* (Desf.) is typical of the Lily family. The upper and lower epidermis has a striated cuticle with one row of short palisade cells, and is isodiametrical. The spongy parenchyma is compact with a few of the cells containing calcium oxalate raphides. Surrounding the vascular bundle is a ring of fibers (in older leaves) and one row of collenchyma cells, particularly at the lower surface of the leaf. Representing the conducting tissues are small tracheae and tiny sieve tubes. The cells of the upper and lower epidermis are elongated with wavy walls, having stomatal apparatuses only on the lower epidermis surrounded by four, or rarely by five, neighboring cells. See Figs. 2 and 3.

Rhizome.—Epidermis consists of elongated cells accompanied by one row of collenchyma. Filling the parenchyma of the pericycle are small starch grains, the smallest being 15 μ; the largest 37.5 μ; with an average of 23.2 μ; and calcium oxalate raphides in bundles from 22.5 to 45 μ with an average of 33.8 μ. The endodermis is slightly lignified, consisting of one or, in some cases, two rows of cells. The tracheae are arranged in groups surrounding the pith. See Fig. 4.

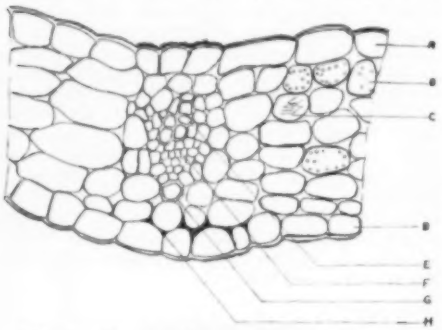

Fig. 2.—Cross section of leaf. *A*, Upper epidermis; *B*, palisade cells; *C*, spongy parenchyma with calcium oxalate raphides; *D*, lower epidermis; *E*, tracheae; *F*, sieve tissue; *G*, bast fiber; *H*, collenchyma.

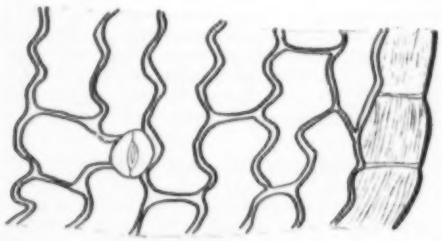

Fig. 3.—Lower epidermis with stoma and striated marginal epidermal cells.

Powdered Root and Rhizome.—The powder is characterized by numerous small starch grains, calcium oxalate raphides, fragments of tracheae, sclerenchyma fibers, and parenchyma.

Powdered Leaf.—The identifying features of the powdered leaf are the calcium oxalate raphides, the characteristic epidermal cells, and the striated cuticle of those epidermal cells at the margin of the leaf. See Fig. 5.

Phytochemistry.—Collections of the whole plant were made in and around the Big Rapids, Michigan area during the latter part of April and May 1957. The material collected was dried at room temperature for three days before grinding in a hammer mill to a fine powder. This powder proved to be very irritating to the nasal mucosa.

Preliminary chemical tests were conducted on water infusions of the material collected and the following results tabulated.

Substance	Reagent	Result
Alkaloids	$AuCl_3$, T. S.	−
	Mayer's reagent	−
	Picric acid T. S.	−
Tannins	$FeCl_3$ T. S.	+
Reducing substances (sugars, glycosides, and saponins)	Fehling's T. S.	+
Saponins	2% Rat blood suspension	+

Since the preliminary tests indicated the presence of saponins, further investigation dealt with the isolation of this type of compound.

Twenty grams of the powdered plant were placed in Soxhlet extraction thimbles and extracted with petroleum ether for forty-eight hours, yielding 0.40 Gm. of a green, waxy, resinous material. (All yields indicated have been evaporated and desiccated to constant weight.) The solvent was then changed to ethyl ether and extraction continued for forty-eight additional hours. This solvent yielded 0.43 Gm. of a green semioily material. The thimbles were then placed in 300 ml. of sterile distilled water containing a small amount of chloroform to retard mold growth. After forty-eight hours of soaking, the thimbles were removed and the solution vacuum filtered through pumice. The filtrate was then passed through 1- × 10-inch columns of Alcoa activated alumina which adsorbed a large portion of the coloring matter and other unwanted impurities. The filtrate obtained was then evaporated without the use of heat in a vacuum desiccator until crystallization took place. The crystals were then filtered out, yielding 0.06 Gm. (0.3%) of a yellow crystalline material plus a yellow amorphous substance. Varying amounts of ethyl alcohol and isopropyl alcohol were passed through the alumina columns following the aqueous extract, in order to determine the extent to which the extract was adsorbed on the alumina.

Attempts to isolate the active principle by extraction with 95% ethanol and subsequent purifica-

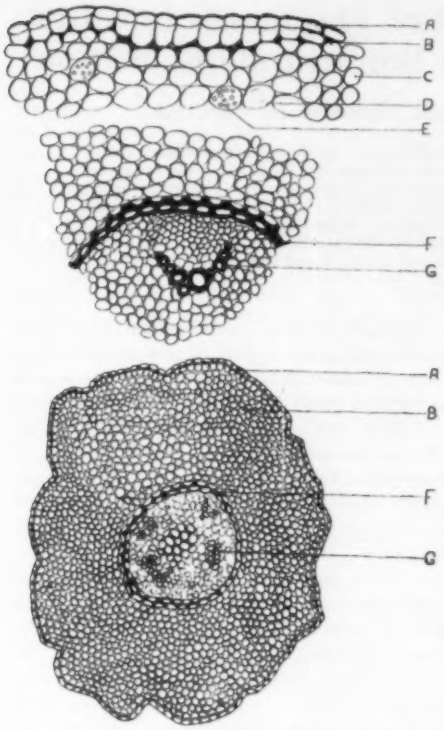

Fig. 4. Top—Cross section of rhizome in high power. *A*, Epidermis; *B*, collenchyma; *C*, pericycle; *D*, parenchyma with calcium oxalate raphides; *E*, parenchyma with starch grains; *F*, endoderm; *G*, tracheae.

Bottom—Cross section of rhizome in low power, *A*, Epidermis; *B*, collenchyma; *F*, endoderm; *G*, tracheae.

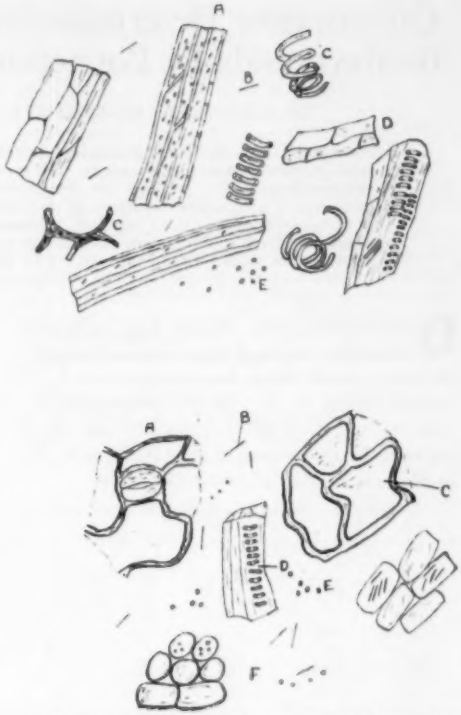

Fig. 5. Top—Powdered root and rhizome. *A*, Fibers; *B*, calcium oxalate raphides; *C*, tracheae; *D*, parenchyma; *E*, starch grains.

Bottom—Powdered leaf. *A*, Lower epidermis with stoma; *B*, calcium oxalate raphides; *C*, upper epidermis; *D*, fragment of vascular bundle; *E*, chlorophyll grains; *F*, lower epidermis with spongy parenchyma.

tion of this extract with lead subacetate were unsuccessful.

Column chromatography was also attempted in the separation of the active constituents from the watery extract, but they were not adsorbed on the column and percolated through it, leaving this method advantageous only in purification.

The two portions isolated were then tested for their hemolytic effect on rat blood. The crystals proved to be some unpure, unidentified substance with no hemolytic properties, but the amorphous substance caused extensive hemolysis.

PRELIMINARY STUDY OF PHARMACOLOGIC ACTIVITY

The water extract was tested for its effect on spontaneous contractions of the isolated ileum of rats and guinea pigs. Doses of 0.5 ml. (50 ml. bath) produced an increase in tone, but had no effect on either rate or amplitude of contractions. Larger doses of 1.0 ml. and 2.0 ml. resulted in a greater increase in tone.

The same extract was tested for its effect on blood pressure. The blood pressure was recorded by direct cannulation of the carotid artery and injections were made into the femoral vein. Average responses were as follows:

Animal	Dose	Effect
Cat	0.2 ml./Kg	No effect
Cat	1.0 ml./Kg.	Transient fall to 80% of normal
Rat	1.0 ml./Kg.	Transient fall to 75% of normal
Rat	2.0 ml./Kg.	Transient fall to 50% of normal

From these results it was impossible to ascertain definite pharmacological properties. Additional pharmacologic investigations are in progress.

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Quantitative Determination of Flavanones in Citrus Bioflavonoids by Potassium Borohydride Reduction*

By KENNETH M. ROWELL and DONALD H. WINTER

Flavanones reduced with potassium borohydride and acidified with hydrochloric acid develop red to violet colors. This reaction forms the basis for a quantitative spectrophotometric method for the determination of flavanones. The procedure outlined is sufficiently sensitive to determine quantitatively solubilized flavanones at concentrations of 0.02 mg./ml. Determinations of flavanone content of typical pharmaceutical formulations containing citrus bioflavonoids demonstrate complete recovery of added bioflavonoids. The method is rapid and the results are reproducible.

DEFINITIVE PAPERS dealing with molecular structure, chemical properties, and biogenesis of flavanones have been written by T. A. Geissman, *et al.* (1, 2). Physiological properties, capillary and stress effects primarily, have been reported by Scarborough and Bacharach (3), Levitan (4), Martin (5), to mention a few.

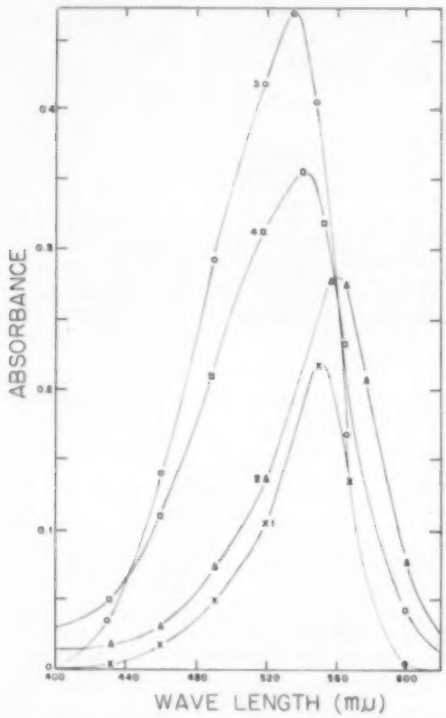

Fig. 1.—Absorption spectra of colored substances formed by reduction and subsequent acidification of the flavanones. (1) Naringin; concn. 2.08 γ/ml., max. 552 mμ; (2) Hesperidin: concn. 4.33 γ/ml., max. 560 mμ; (3) Naringenin: concn. 2.08 γ/ml., max. 535 mμ; and (4) Hesperetin: concn. 2.08 γ/ml., max. 540 mμ. Solvent: Acid reagent, 10-mm. Pyrex cells used.

* Received October 21, 1958, from the Orange Products Division, Sunkist Growers, Ontario, Calif.

Methods for quantitative estimation of flavanones, chief group of bioflavonoids in citrus, have been generally unsatisfactory. The gravimetric as well as previous colorimetric methods lack specificity and convenience especially when applied to finished pharmaceuticals containing small amounts of flavanones compared to other ingredients. Colorimetric methods based on the cyanidin reaction (1) appeared to warrant careful consideration. Earlier attempts to adapt this reaction to the quantitative determination of flavanones have been of limited utility because of the difficulty in controlling the large amount of heat generated during magnesium reduction, with consequent variation in the colors produced. It was, therefore, desirable to find a better method of reduction of the flavanones with controlled color development.

In recent years the complex saline hydrides have become commercially available and have been shown to possess specific reducing properties (6, 7). Bauer, Birch, and Hillis (8) reported the reduction of flavanone with sodium borohydride yielded β 4-chromanol, m. p. 146°. These investigators also reported the flavonols rutin and quercetin were not reduced by sodium borohydride. Horowitz (9) tested many flavonoids and found only the flavanones produced red to violet colors when reduced with $NaBH_4$ and acidified with hydrochloric acid. He used $NaBH_4$ followed by hydrochloric acid as a spray reagent to locate flavanone spots separated by paper chromatography.

EXPERIMENTAL

Selection of Reducing Agent.—Preliminary experimentation with sodium borohydride and potassium borohydride for the reduction of flavanones in alcoholic and aqueous solutions showed that little heat was liberated during the reduction. It was also found that reduction to form a stable colorless compound could be accomplished by a noncolor-producing acid, e. g., acetic acid, enabling a final well-controlled color development with hydrochloric acid. Because potassium borohydride is non-

hygroscopic and reacts less vigorously in an aqueous system than sodium borohydride, it proved the more satisfactory metallic hydride. In addition, uniform results were obtained using various lots of potassium borohydride.

Apparatus.—Absorbance measurements were made with a Beckmann model B spectrophotometer using 10-mm. Pyrex cells. Glassware used consisted of Normax volumetric pipets and flasks, Pyrex test tubes (20 × 150 mm.) with polyethylene stoppers. A water bath with a temperature 20° ± 0.5° was used for the adjustment of volumes and temperature control.

Reagents.—Potassium borohydride, 97+%, (Metal Hydrides Co., Beverly, Mass.). "Acid reagent": prepared by diluting 5 ml. of hydrochloric acid, (reagent, A. C. S.) to 100 ml. volume with glacial acetic acid, (reagent, A. C. S.). Sodium hydroxide solution, 0.1 *N*. Acetic acid solution, 0.5 *N*.

Preparation of Standard Flavanone Samples.—Flavanone glycosides and their corresponding aglycones were purified from commercially available materials by appropriate procedures.

Hesperidin (*Hesperetin-7-rhamnoglucoside*).—Colorless needles melting at 261–263° with decompn. (10, 11), gravimetric purity 98+%.

Hesperetin (*3', 5, 7,-Trihydroxy-4'-methoxy Flavanone*).—Colorless plates melting at 232–234° with decompn. (12).

Naringin Dihydrate (*Naringenin-7-rhamnoglucoside*).—Colorless needles melting at 170–172° with decompn. (13), gravimetric purity 98+%.

Naringenin (*4', 5, 7-Trihydroxy Flavanone*).—Colorless needles melting at 249–251° with decompn. (13).

ANALYTICAL PROCEDURE

Preparation of Flavanone Solutions.—Weigh 50 mg. of the flavanone (100 mg. in the case of hesperidin) into a 100-ml. volumetric flask. Solubilize the flavanone by adding 10 ml. of 0.1 *N* sodium hydroxide. Adjust the volume to 100 ml. with distilled water. Prepare final solution by diluting 1:10 for hesperidin; 1:20 for hesperetin and naringin; 1:40 for naringenin, with distilled water.

Reduction. Pipet 5 ml. of the final diluted flavanone solution into a test tube (20 × 150 mm.) containing 50 mg. of potassium borohydride. When the potassium borohydride has dissolved, add dropwise 5 ml. of 0.5 *N* acetic acid to reduce the flavanone.

Color Development.—Into a polyethylene-stoppered test tube pipet 5 ml. of "acid reagent." Pipet 1 ml. of the reduced flavanone material into the "acid reagent" tube. Shake and place in the 20° water bath. The color which develops is read against a blank prepared just prior to reading and consists of 1 ml. of the reduced material plus 5 ml. of glacial acetic acid. It is necessary to prepare the blank just prior to reading because the reduced aglycones produce a small amount of color upon long standing in glacial acetic acid. The holding time for color development varies with the flavanone being determined. When assaying hesperidin, the maximum color is read at eighteen minutes; for naringin and naringenin, thirty minutes; and hesperetin five minutes. Transfer the colored solution just before the maximum intensity has been reached and record maximum absorbance at the predetermined wavelength for the flavanone being assayed. See Figs. 1 and 2.

Adherence to Beer's Law.—Beer's law is in effect from the concentrations 0 to 25 mcg. per ml. for hesperetin, naringin, and naringenin. A good linear relationship is shown for hesperidin from 0 to 100 mg. per ml. concentration, see Fig. 3.

Sensitivity.—Quantities of flavanones as low as 5 mcg. per ml. of solution (30 mcg. per ml. of hesperidin solution) can be determined by the potassium borohydride reduction method, see Fig. 3. The detectability level of the magnesium-hydrochloric acid reduction method is about 100 mcg. per ml. of ethanol (1). Thus, it appears that this test could be used to measure the amount of chromatographically isolated flavanones.

Reproducibility.—The reproducibility of results on several flavanones is shown in Table I. The absorbance values were obtained on individually reduced samples and illustrate the reproducibility of both the reduction and the color development.

Statistically, the standard deviations for the

TABLE I.—ABSORBANCE VALUES ON REPLICATE ANALYSES

Test No.	Naringenin	Hesperetin	Hesperidin	Naringin
1	0.260	0.375	0.300	0.224
2	0.258	0.372	0.300	0.225
3	0.260	0.375	0.299	0.223
4	0.260	0.372	0.299	0.222
5	0.261	0.378	0.298	0.222
6	0.259	...	0.299	...

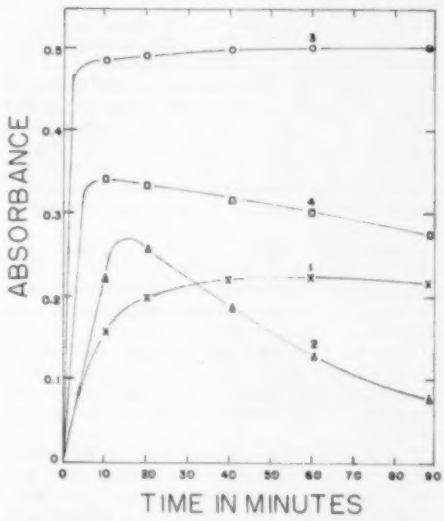

Fig. 2.—Formation and stability of color at 20°. (1) Naringin: concn. 2.08 γ/ml., max. color and stability, sixty min.; (2) Hesperidin: concn. 4.33 γ/ml., max. color and stability, eighteen to twenty min.; (3) Naringenin: concn. 2.08 γ/ml., max. color and stability, sixty min.; and (4) Hesperetin: concn. 2.08 γ/ml., max. color and stability five to six min. Solvent: Acid reagent, 10-mm. Pyrex cells used.

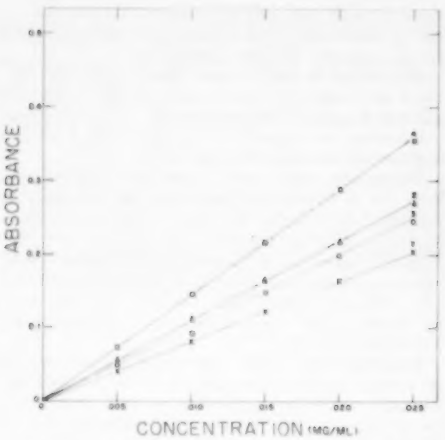

Fig. 3.—Adherence to Beer's law. The concentration of hesperidin is four times that indicated, (1) Naringin, (2) Hesperidin, (3) Hesperetin, and (4) Naringenin.

four flavanones were not significantly different so they were pooled to give an estimated standard deviation per determination of 0.0014 (absorbance). This does not include weighing errors. This is considerably less than the error for other methods used to determine flavanones quantitatively.

Factors Influencing Color Development.—*Rate of Reduction.*—The best replication was obtained by adding the acetic acid in the following manner: The tube containing the solution to be reduced was placed over a magnetic stirrer and a Teflon-covered stirring bar was inserted. While the solution was being stirred rapidly, the acetic acid was allowed to drop in slowly. In order to control the drop rate a fine capillary was used on the top of the pipet which changed its flow rate to eight minutes, see Fig. 4. After four minutes, at which time most of the acetic acid had drained, the capillary was removed and the last amount in the pipet was allowed to drain normally.

Temperature.—Different temperatures during the color development step greatly influence the rate and intensity of the color development. Figure 5 uses hesperidin as an example to illustrate the time required to attain maximum absorbance at 0, 20, and 40°. As 20° allows ample time before maximum color occurs and yet is not excessive, this temperature was selected. However, lower temperatures may be employed to produce greater stability.

Acid Concentration.—As the ratio of the hydrochloric acid to the acetic acid is increased in the "acid reagent," the color intensity increases. However, a larger proportion of hydrochloric acid causes the color to become less stable. In order to obtain a moderate degree of color stability and yet retain sensitivity, the ratio of hydrochloric acid to acetic acid was empirically chosen. When using the potassium borohydride method qualitatively, the sensitivity can be greatly increased by larger amounts of hydrochloric acid in the "acid reagent."

Stability of Color.—Figure 2 shows the development and decay of color intensity at 20°. Since naringin and naringenin color is quite stable after thirty minutes development, they are read at thirty minutes. Hesperidin and hesperetin are read, preferably, at eighteen to twenty minutes and five to six minutes, respectively.

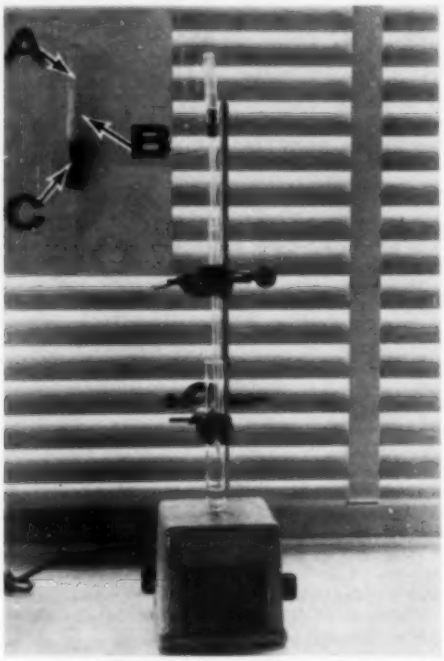

Fig. 4—Apparatus for controlled reduction with acetic acid. A, capillary; B, glass tube with glass wool packing to protect capillary; and C, rubber sleeve for attachment of capillary to pipet.

Stability of Flavanones in Final Dilute Alkaline Solution.—The flavanones will decompose slightly in alkaline solutions when held at 20° for several hours. It is, therefore, necessary that the holding time before reduction be as short as practical. See Table II.

TABLE II.—STABILITY OF FLAVANONES IN ALKALINE SOLUTION

Flavanone	Absorbance Readings 15 min.	60 min.	300 min.
Hesperidin	0.270	0.270	0.259
Hesperetin	0.363	0.360	0.360
Naringin	0.210	0.200	0.195
Naringenin	0.235	0.235	0.230

Each alkaline solution was held at 20° for the designated time and the reduction and color formation steps followed as outlined under procedure. Since the samples used were not anhydrous, higher values would have resulted if the samples had been dried and handled carefully so as to avoid moisture absorption.

Absorptivity Values on Anhydrous Flavanones.[1]—

[1] The term anhydrous flavanones used here pertains to materials dried for two hours at 105°.

TABLE III.—ANALYSIS FOR HESPERIDIN COMPLEX IN FINISHED PHARMACEUTICALS

Preparation	Hesperidin Complex[d] added to each tablet, mg.	Recovery,[e] %	Calcd. Amount Determined, mg.
Cold tablet[a]	20	103.6, 99.4, 101.8	20.7, 19.9, 20.9
	10	99.4, 102.4, 100.6	9.9, 10.2, 10.1
Multivitamin[b]	20	99.4, 102.0, 106.0	19.9, 20.4, 21.2
	10	103.0, 104.0, 104.8	10.3, 10.4, 10.5
Liver and iron[c]	20	104.3, 104.2, 105.3	20.9, 20.8, 21.1
	10	103.2, 103 0, 97.4	10.3, 10.3, 9.7

[a] Contains thonzylamine·HCl, aspirin, phenacetin, caffeine, and ascorbic acid.
[b] Commercial multivitamin and mineral preparation.
[c] Commercial liver and iron preparation containing, in addition, copper, B_{12}, and ascorbic acid.
[d] Orange Products Division, Sunkist Growers, Ontario, Calif.
[e] Absorbance of test/absorbance of control × 100 = per cent recovery.

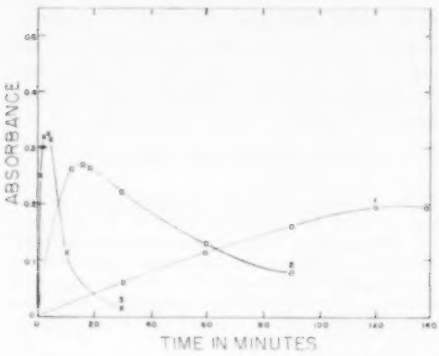

Fig. 5.—The intensity and stability of the color developed by reduced hesperidin at different temperatures: (1) 0°, (2) 20°, and (3) 40°.

The following list represents the absorptivity values obtained on the purified materials used in this study: hesperidin, (a) = 34,000; hesperetin, (a) = 181,000; naringin·$2H_2O$, (a) = 114,000; naringenin, (a) = 250,000.

More effective methods of purification may result in higher values. However, the authors have been unable to obtain materials from any source with absorptivity values higher than these.

Application of the Method to Finished Pharmaceuticals.—Many preparations containing hesperidin plus ascorbic acid and excipients may be analyzed as under "Analytical Procedure." Complex preparations may be successfully analyzed by removing the water solubles before analysis. The following technique has proved to be adequate with all preparations tested to date.

Procedure.—Grind the finished pharmaceutical to pass through a 100-mesh screen. Weigh sufficient material to contain 20 mg. of the hesperidin product used in the preparation. Weigh 20 mg. from the lot of hesperidin used to serve as a control. Place each sample in a 50-ml. centrifuge tube and add 20 ml. of water which was previously saturated with hesperidin to which a small amount of antifoam (1 mg./100 ml. Dow-Corning AF) has been added. Mix and centrifuge at 2,000 r. p. m. for five minutes. Examine centrifuged tubes for floating particles—especially in the control, and recentrifuge if necessary. Decant carefully and repeat washing a second time. Decant and solubilize the hesperidin by adding 10 ml. of 0.1 *N* NaOH and transfer, quantitatively, to a 25-ml. volumetric flask. Dilute to volume and filter. Dilute the filtrate 1:10. Reduce this dilution and proceed as under "Analytical Procedure." The absorbance value of the test sample is compared to that of the control and the per cent recovery calculated.

Table III illustrates the precision obtained from three complex preparations. The results are of triplicate analyses. When the pharmaceuticals themselves, without hesperidin, were tested none showed even a trace of flavanone content.

SUMMARY

The potassium borohydride method for flavanones offers a rapid, simple, quantitative means of determining these compounds and has the following distinct advantages over previous methods: (*a*) Only with flavanones are red to violet colors produced, (*b*) Amounts as low as 5 mcg. per ml. can be determined quantitatively (Hesperidin 20 mcg./ml.), and (*c*) The reduction may be conducted without external cooling. For the past two years this method has been used in connection with other methods to determine the flavanone content in hesperidin, naringin, naringenin, and hesperetin products. Consistent results have been obtained and considerable time saved in analysis. The method has been shown to be adaptable to hesperidin analyses in complex pharmaceutical preparations.

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The Synthesis of Some Derivatives of Cinnamic Acid and Their Antifungal Action*

By H. WAYNE SCHULTZ† and GAIL A. WIESE

Various amide derivatives of *o*-chlorocinnamic acid, *p*-nitrocinnamic acid, and cinnamic acid were prepared and tested for antifungal activity against four organisms. N-(4-Chlorophenyl)-2-chlorocinnamamide proved to be the most active compound against *M. gypseum* and *T. rubrum*, while N-(3-chlorophenyl) cinnamamide proved to be the most active compound against *M. canis* and *T. mentagrophytes*. In general, the results of the test indicated that most of the cinnamamide derivatives had little or no antifungal activity *in vitro*.

AROMATIC COMPOUNDS represent a class of antifungal agents that have been successfully used in treating superficial mycoses. In reviewing the literature concerning these compounds it was noted there have been few investigations directed towards the study of antifungal activity of cinnamic acid derivatives. It was thought that these derivatives might possess antifungal activity because:

(*a*) Cinnamic acid and its derivatives have been reported to have antibacterial, antitubercular, and antifungal activity. In regards to antifungal activity, Uppal (1) found cinnamic acid to be more fungicidal than benzoic acid, while having approximately the same activity as salicylic acid. Also, *o*-chlorocinnamic acid and *p*-nitrocinnamic acid have been reported to have antifungal activity (2).

(*b*) The relationship of cinnamic acid to fungistatic benzoic acid derivatives. Benzoic acid, of which cinnamic acid is a vinylog, and its derivatives have received considerable investigation as antifungal agents. One of these derivatives, 2-*n*-amyloxybenzamide, has been found to be more effective than many of the commonly used antifungal agents. Also, various salicylic acid derivatives, such as salicylanilide (3) and the phenylsalicylamides (4) have been reported to have therapeutic value as antifungal agents.

(*c*) The presence of alpha-beta unsaturation in the molecule. Several investigators have reported increased antifungal activity in compounds due to the presence of alpha-beta unsaturation. Of further significance, cinnamic acid represents the beta-phenyl derivative of acrylic acid, and various derivatives of this acid have been found to have antifungal activity.

Since amide derivatives of other related acids, such as salicylic acid and acrylic acid, have shown useful antifungal properties, the cinnamamide derivatives were selected to be prepared and tested. Furthermore, a review of the literature revealed that amides of cinnamic acid have not been investigated for antifungal activity.

EXPERIMENTAL PART I

The following processes exemplify the various methods used for the preparation of the cinnamic acid derivatives. The *o*-chlorocinnamoyl chloride was prepared from *o*-chlorocinnamic acid[1] by the method as recorded by Andrews, Van Campen, and Schulman (5). Information concerning the various cinnamamides which were prepared is summarized in Table I.

Method I.—N - (2 - Chlorophenyl) - 2 - chlorocinnamamide.—In a 500-ml. round-bottom flask, equipped with a reflux condenser, was placed 10 Gm. (0.05 mole) of *o*-chlorocinnamoyl chloride. A solution of 6.4 Gm. (0.05 mole) *o*-chloroaniline, dissolved in 30 ml. of C. P. grade pyridine, was added slowly to this. The reaction mixture was refluxed for three hours and then poured slowly, with stirring, into a beaker containing 300 ml. of distilled water. The water solution was cooled and the crystalline material removed with suction filtration and dried. The product was purified by recrystallization from ethyl alcohol. The yield was 7.9 Gm. (54.1% of theoretical) of white crystals which melted at 170.5 to 171.5°.

Method II.—N - (2 - Chlorophenyl) - 4 - nitrocinnamamide.—In a 500-ml. round-bottom flask was placed 20 Gm. (0.1 mole) of *p*-nitrocinnamic acid and 50 ml. benzene. The mixture was cooled in an ice bath and 19.2 Gm. (0.16 mole) of thionyl chloride, dissolved in 15 ml. benzene, was added, dropwise. The reaction mixture was refluxed for one and one-fourth hours, after which time complete solution was effected. The excess thionyl chloride was removed by distillation under reduced pressure. Two fresh portions of 50 ml. benzene were added and distilled from the acid chloride to facilitate removal of the thionyl chloride. To the resulting acid chloride, dissolved in 75 ml. benzene, a solution of 26.5 Gm. (0.21 mole) of *o*-chloroaniline in 50 ml. benzene was added, dropwise. The reaction mixture was refluxed for one-half hour, after which the solvent was removed by evaporation. The resulting product was recrystallized from methyl ethyl ketone. The yield

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[1] The *o*-chlorocinnamic acid used in this study was supplied through the courtesy of the Hayden-Newport Chemical Corp., New York, N. Y.

TABLE I.

Compound	Method	Yield, %	M. p., °C.	Recrystn. Solvent	N, % Calcd.	N, % Found
N-(2-Chlorophenyl) cinnamamide	I	45.8	137–138	EtOH	5.44	5.39
N-(3-Chlorophenyl) cinnamamide	I	27.9	125–126	EtOH	5.44	5.62
N-(4-Chlorophenyl) cinnamamide	I	59.4	185–186	EtOH	5.44	5.40
N-(2,4-Dichlorophenyl) cinnamamide	I	35.8	162–164	EtOH	4.79	4.89
N-(2,5-Dichlorophenyl) cinnamamide	I	22.2	177–178	EtOH	4.79	4.83
N-(2-Benzothiazyl) cinnamamide	I	47.4	245–246	EtOH-acetone	10.00	9.84
N-(3-Quinolyl) cinnamamide	I	61.0	217–218	EtOH	10.21	10.17
N-(5-Chloro-2-pyridyl) cinnamamide	I	59.2	186–187	EtOH	10.82	10.63
N-(2-Methyl-4-chlorophenyl) cinnamamide	I	29.4	189–190	EtOH	5.16	4.96
N-(2-Chlorophenyl)-2-chlorocinnamamide	I	54.1	170.5–171.5	EtOH	4.80	4.70
N-(3-Chlorophenyl)-2-chlorocinnamamide	I	77.4	162–163	Benzene	4.80	4.71
N-(4-Chlorophenyl)-2-chlorocinnamamide	I	76.7	190–190.5	Chloroform	4.80	4.74
N-(2,4-Dichlorophenyl)-2-chlorocinnamamide	I	69.2	177–178	CCl_4	4.29	4.21
N-(2,5-Dichlorophenyl)-2-chlorocinnamamide	I	76.0	158–159	CCl_4	4.29	4.19
N-(2-Benzothiazyl)-2-chlorocinnamamide	I	86.8	240–241	Me cellosolve	8.91	8.71
N-(3-Quinolyl)-2-chlorocinnamamide	I	62.2	213–214.5	EtOH	9.08	8.87
N-(5-Chloro-2-pyridyl)-2-chlorocinnamamide	I	54.6	231–232	EtOH	9.56	9.39
N-(2-Methyl-4-chlorophenyl)-2-chlorocinnamamide	I	54.5	291–292	EtOH	4.58	4.76
N-(2-Chlorophenyl)-4-nitrocinnamamide	II	66.1	220–221	Me Et ketone	9.26	8.99
N-(3-Chlorophenyl)-4-nitrocinnamamide	III	68.9	196–197.5	EtOH	9.26	8.94
N-(4-Chlorophenyl)-4-nitrocinnamamide	III	60.4	226–227	EtOH-acetone	9.26	8.96
N-(2,4-Dichlorophenyl)-4-nitrocinnamamide	III	63.3	226–227	EtOH	8.31	8.12
N-(2,5-Dichlorophenyl)-4-nitrocinnamamide	III	59.0	251–253	Pyridine	8.31	8.06
N-(2-Benzothiazyl)-4-nitrocinnamamide	III	47.6	320–321	Pyridine	12.63	12.64
N-(3-Quinolyl)-4-nitrocinnamamide	III	51.6	313–314	Pyridine	13.17	12.89
N-(5-Chloro-2-pyridyl)-4-nitrocinnamamide	III	43.8	262–264	Pyridine	13.85	13.56
N-(2-Methyl-4-chlorophenyl)-4-nitrocinnamamide	III	49.7	260–261	Pyridine-EtOH	8.85	8.55

was 20.0 Gm. (66.1% of theoretical), of light yellow crystals which melted at 220 to 221°.

Method III.—N - (4 - Chlorophenyl) - 4 - nitrocinnamamide.—In a 500-ml. round-bottom flask was placed 16.1 Gm. (0.083 mole) of *p*-nitrocinnamic acid and 50 ml. benzene. The mixture was cooled in an ice bath and 10.9 Gm. (0.09 mole) of thionyl chloride, dissolved in 15 ml. benzene was added dropwise. The reaction mixture was refluxed for three hours, after which time complete solution was effected. The excess thionyl chloride was removed by distillation under reduced pressure. In order to facilitate the removal of thionyl chloride, 25 ml. toluene was added and distilled from the acid chloride. This purification process was repeated using 25 ml. benzene. To the resulting acid chloride, dissolved in 50 ml. benzene, a solution of 11.6 Gm. (0.09 mole) *p*-chloroaniline in 25 ml. benzene and 25 ml. ether was added, dropwise. The reaction mixture was refluxed for one hour, after which the solvent was removed by evaporation. The product was purified by grinding it into a fine powder, and washing it with two 100-ml. 2% hydrochloric acid, two 100-ml. water, two 100-ml. 2% sodium carbonate, and two 100-ml. water. The resulting product was recrystallized from equal parts of ethyl alcohol and acetone. The yield was 16.2 Gm. (60.4% of theoretical) of light yellow crystals, which melted at 226 to 227°.

EXPERIMENTAL PART II

The compounds were tested for antifungal activity using a modified paper disk technique similar to that described by Kligman and Rosenweig (6). The test organisms used were: *Microsporum canis*, *Microsporum gypseum*, *Trichophyton rubrum*, and *Trichophyton mentogrophytes*. Acetone solutions of the compounds were prepared in concentrations of 4, 2, 1, and 0.5%. Saturated solutions were prepared for compounds whose solubility in acetone were less than 0.5%. Two known antifungal agents, undecylenic acid and salicylanilide, were tested as reference compounds.

Sterile filter paper disks (12.7 mm. in diameter) were immersed for thirty seconds in each of the acetone solutions of the compounds. The excess liquid was drained off by touching the disk to the wall of the container, and the disk was placed immediately on the agar surface of a Petri dish which had been previously inoculated with the desired test organism. On each Petri dish were placed two filter disks saturated with the acetone solution. After incubation for fifteen days at room temperature, the zones of inhibition, representing the minimum distance between the periphery of the filter disk and the colony growth, were recorded as the mean of the two measurements. Only those compounds which had a zone of inhibition greater than 3 mm. are given in Table II.

DISCUSSION

In the separation and purification of the *p*-nitrocinnamic acid derivatives, it was necessary to wash the crude product with a dilute hydrochloric acid solution first, in order to remove the excess amine, and then to wash the remaining product well with dilute sodium hydroxide solution, to remove the excess *p*-nitrocinnamic acid. This procedure was

TABLE II.—MINIMUM WIDTH[a] OF ZONES OF INHIBITION, IN MILLIMETERS

Compound	w/v, %	*M. canis*	*M. gypseum*	*T. rubrum*	*T. mentagrophytes*
N-(2-Chlorophenyl) cinnamamide	0.5	0	0.5	0	0
	1.0	0	1	0	0
	2.0	0	2	0	0
	4.0	1	3.5	1	0
N-(3-Chlorophenyl) cinnamamide	0.5	1	5	1	0
	1.0	2.5	6.5	1	0.5
	2.0	4.8	7	1	9
	4.0	7	8	1	11.5
N-(3-Chlorophenyl)-2-chlorocinnamamide	0.5	1	2	0	1
	1.0	1.5	3	1	1.5
	2.0	3.5	3.5	3	2.5
	4.0	5	5	4	4.5
N-(4-Chlorophenyl)-2-chlorocinnamamide	0.5	0	6.5	0.5	0
	1.0	0	8.5	1.5	0
	2.0	0	10	2.5	0
	4.0	1.5	24	10	0
N-(2,5-Dichlorophenyl)-2-chlorocinnamamide	0.5	0	1.5	0	0
	1.0	2	2	0	0
	2.0	3	3	0	0
	4.0	...[b]	...[b]	...[b]	...[b]
Salicylanilide	0.5	9	9.5	7.5	6
	1.0	14	10.5	9	14
	2.0	20	14	10	18
	4.0	24	19	12.5	22.5
Undecylenic acid	0.5	4	10.5	7	5
	1.0	6.5	11.5	11	11
	2.0	10	16	14.5	16
	4.0	14	25	20	20
Acetone	0.5	0	0	0	0
	1.0	0	0	0	0
	2.0	0	0	0	0
	4.0	0	0	0	0

[a] Average of two measurements. [b] Insoluble in acetone at the stated concentration.

necessitated due to the low solubility of the amides in organic solvents, as well as the high degree of insolubility of *p*-nitrocinnamic acid (a contaminant) in the solvents.

It was desired in this investigation to test and compare the compounds at various concentrations to determine the effect of concentration on antifungal properties. Because of the insoluble nature of most of the compounds, particularly the *o*-chloro and *p*-nitro derivatives, it was not possible to test all of the compounds at several different concentrations. However, it was decided to prepare and test saturated solutions of these "insoluble" compounds to determine if any of these compounds would exert antifungal activity when at a concentration which was equal to their maximum solubility, i. e., a saturated solution.

The results of the test indicated that most of the cinnamamide derivatives had little or no antifungal activity *in vitro*. N(4-Chlorophenyl)-2-chlorocinnamamide proved to be the most active of the compounds tested against *M. gypseum*, having activity greater than salicylanilide while being comparable to undecylenic acid at 4% concentration. Also, it was the most active of the compounds against *T. rubrum*, having activity less than salicylanilide and much less than undecylenic acid. Against *M. canis* and *T. mentagrophytes*, N-(3-chlorophenyl) cinnamamide proved to be the most active compound. In general, it appeared that substitution on either the phenyl ring of cinnamic acid or variation of the amine portion of the molecule had little effect on antifungal activity of these compounds.

The apparent lack of antifungal activity as shown by the tested compounds may be due to several factors. Perhaps, chief among these factors would be the general insolubility (both in water and organic solvents) that was exhibited by these compounds. Also, it may be possible that the testing procedure disguised the true antifungal activity of these compounds. Since the zones of inhibition are greatly dependent upon the ability of compounds to diffuse in the agar, the diffusability factor, rather than the inhibitory factor may have been measured. Although the testing method used represents a useful screening procedure for antifungal activity, it may not correlate the *in vitro* results to clinical effectiveness.

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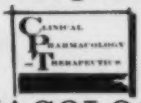
find 
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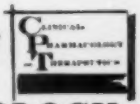
His files of 
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